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Editor
ROY WALDO MINER

PAUL EHRLICH CENTENNIAL

BY

ROBERT J. SCHNITZER AND E. GRUNBERG (*Conference Co-Chairmen*), J. A. AESCHLIMANN, H. BAUER, C. H. BROWNING, H. EAGLE, N. C. FOOT, J. A. KOLMER, H. L. KREKELER, P. REZNIKOFF, C. P. RHOADS, B. SCHICK, AND E. WITEBSKY.

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E. GRUNBERG

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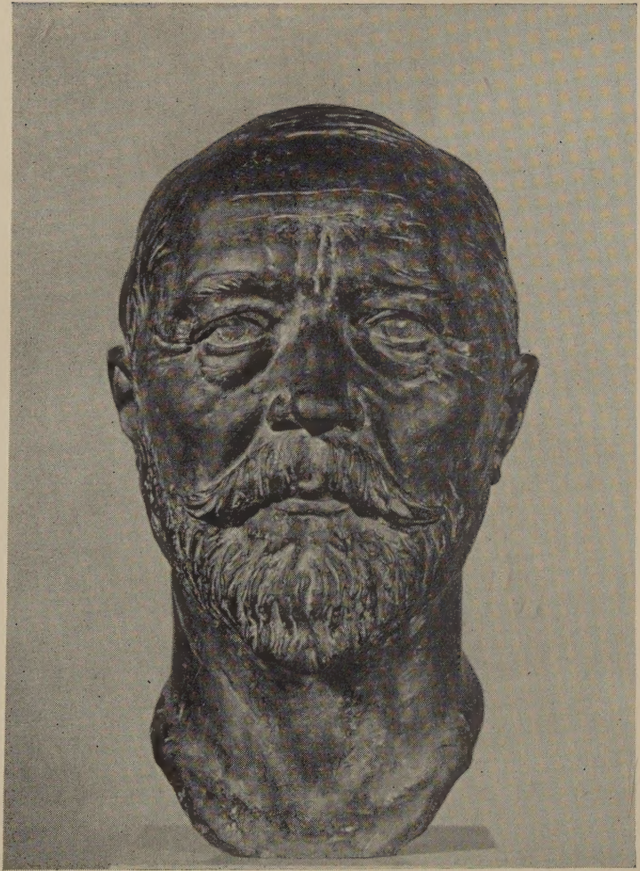

EMIL VON BEHRING (1854–1917)

PAUL EHRLICH (1854–1915)

These portrait busts of Paul Ehrlich and Emil von Behring were presented to The New York Academy of Sciences by His Excellency Heinz L. Krekeler

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PAUL EHRLICH CENTENNIAL*

Conference Co-Chairmen: R. J. SCHNITZER AND E. GRUNBERG
Consulting Editor: E. GRUNBERG

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* This series of papers is the result of a conference held in honor of the centennial of the birth of Paul Ehrlich, by the Section of Biology of The New York Academy of Sciences, March 13, 1954.

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OPENING REMARKS

By John A. Aeschlimann
Hoffmann-La Roche, Inc., Nutley, N. J.

The New York Academy of Sciences is now in session to commemorate tomorrow's centenary of the birth of Paul Ehrlich, one of the earliest workers in chemotherapy. Permit me to read to you seven verses from the 44th chapter of the Book of Ecclesiastes:

"Let us now praise famous men;
and our fathers that begat us,
The Lord manifested in them great glory,
even his mighty power from the beginning.
Such as did bear rule in their kingdoms,
and were men renowned for their power,
giving counsel by their understanding,
such as have brought tidings in prophecies:
leaders of the people by their counsels,
and by their understanding men of learning for the people;
wise were their words in their instruction:
all these were honoured in their generations,
and were a glory in their days.
There be of them, that have left a name behind them,
to declare their praises.
And some there be, which have no memorial;
who are perished as though they had not been,
and are become as though they had not been born."

In choosing to read this passage to you I am mindful that the organizers of this conference have selected, as speakers, some of Ehrlich's co-workers, and in honoring Paul Ehrlich we are also remembering them as contributors to his fame. These speakers *also* are "famous men" whom we have met to praise. Furthermore let us remember the less famous of Ehrlich's assistants, although it would *not* be true to say that they "have no memorial." Scientists *live* in their publications, read by subsequent generations, and do *not* "perish as though they had not been born." So *you*, members of the audience, have already honored Ehrlich by choosing to extend and develop the field of chemotherapy, and though the New York Academy of Sciences may not celebrate your centenaries (I say "may not" rather than "*will* not"), yet it may be said of your researches

"So long as men can breathe, or eyes can see,
So long lives this and this gives life to thee."

The first ceremony of the commemoration is the presentation of a bronze bust of Paul Ehrlich and one of Emil von Behring, who was also born in March 1854, from the Farbwerke Hoechst A. G., Frankfurt, Germany, to the New York Academy of Sciences. The presentation will be made for Hoechst by His Excellency, Doctor Heinz L. Krekeler, Ambassador of the Federal Republic of

Germany, at Washington, D. C., to the organizers of the conference, Doctors R. J. Schnitzer and E. Grunberg, who will give them to the President of the Academy, Doctor Paul Fejos, who, in turn, will accept them in the name of the Academy.

Doctor Heinz L. Krekeler

Doctor Heinz L. Krekeler was born in 1906 in Westphalia and studied physical chemistry at the Universities of Freiburg, Munich, Goettingen, and Berlin, where he received his Ph.D. in 1930. During the next 15 years he was employed by various chemical firms in research, production, and administration. Since World War II, he has been a director and partner in the textbook publishing firm of F. Eilers of Bielefeld, Germany, and Chairman of the Board of Apollinaris Brunnen, A. G.

Doctor Krekeler entered politics for the first time following the war and was selected to be a member of the diet of Lippe in 1946 and, in 1947, was elected to the Parliament of the State of North Rhine Westphalia. In 1949, he served as a member of the German Federal Assembly which elected the first President of the Federal Republic.

As a result of his labors for Germany's democratic reconstruction, Doctor Krekeler was invited to join the foreign service as a career officer, whereupon he divorced himself from all commercial activities.

He took office as Consul General of the Federal Republic of Germany in New York in June 1950. Since that time, he has been the ranking German foreign service officer in this country and was appointed Chargé d'Affaires of the Federal Republic of Germany to the United States on July 2, 1951.

He signed the Geneva Convention on the legal position of refugees, on behalf of Germany, in November 1951 and, in 1952, engaged in negotiations which led to German partnership in the International Monetary Fund, and the Bank for Reconstruction and Development in Washington.

Doctor Heinz L. Krekeler was appointed Ambassador of the Federal Republic of Germany to Washington at the end of June, 1953.

I have pleasure in introducing to you His Excellency, the Ambassador to the United States of America representing the Federal Republic of Germany, Doctor Heinz Krekeler.

ADDRESS AND PRESENTATION OF BUSTS OF PAUL EHRLICH AND EMIL VON BEHRING

By His Excellency Heinz L. Krekeler
Ambassador to the United States of the Federal Republic of Germany

I am deeply honored to be with you today and to have the privilege of presenting to The New York Academy of Sciences the bronze busts of two of my distinguished countrymen, Paul Ehrlich and Emil von Behring.

You are gathered here to commemorate the memory of Paul Ehrlich. I hope that you will not feel it is an intrusion if I present the busts of these two eminent scientists simultaneously. There is far more than a casual coincidence involved. A strong personal bond of friendship existed between these two men, born a day apart—Ehrlich on March 14 and Behring on March 15—one hundred years ago. Both entered the field of science and the special field of biology and so became disciples of the great Robert Koch. They collaborated in their research on serums to produce immunization from infectious diseases. Both reached fame in their chosen fields and were awarded Nobel prizes.

These busts are the gift of the Farbwerke Hoechst of Frankfort-on-the-Main, a German firm which helped bring the great discoveries of Ehrlich and Behring to practical application. Their presence in The New York Academy of Sciences is an eloquent testimonial of the ties of friendship that have happily been reestablished between the United States and the Federal Republic of Germany.

Today there will be several speakers who will talk to you about the scientific discoveries and contributions of Paul Ehrlich. I think it would be proper, therefore, for me to speak to you in more general terms about this great personality and what his ideas may mean for us in this era.

Paul Ehrlich began his life in a small town in Silesia, and, before his death in 1915, he had attained the highest honors which were within the reach of a scientist of his generation. He was a member of numerous German and foreign academies and scientific societies. He was the recipient of a series of foreign and German orders and medals of distinction, and the holder of several honorary doctor's degrees, including those of the universities of Chicago and Oxford. He also was awarded five honorary prizes, of which I should like to mention especially the Nobel Prize for Medicine, which he received in 1908, together with Professor Metchnikoff of the Institute Pasteur in Paris, for studies in immunity.

How did Ehrlich make his way from boyhood in a remote town in the eastern part of Germany to the position of a top-ranking scientist of his time? From what we know about his life, this development was owing to a combination of his environment and character. We know that his maternal grandfather made natural science a hobby and delivered speeches and held discussion evenings on general science subjects for the citizens of his native town. Furthermore, Ehrlich's grandfather owned a comparatively comprehensive library on various fields of natural science. This library continuously attracted the attention of Paul Ehrlich as a young high-school student.

But this environment alone would not have led Ehrlich to his achievements. Much of the answer lies in Ehrlich's own character, in his passionate desire to search out the truth, and in his deep-rooted curiosity. It is the desire for the truth, the urge and ability to learn, and the readiness to devote one's entire life without reservation to the task, that characterize an outstanding scientist. These qualities apply fully to Paul Ehrlich. In the field of medicine, he was possessed by a heartfelt desire to help and to have a share in delivering mankind from the various diseases and their after-effects for which no remedies were known at the time.

We find evidence of Ehrlich's searching mind and drive for knowledge in his early days as a medical student. Not even the long lectures and practical laboratory work at the various universities could quench his thirst for answers to research problems. Instead of turning to rest and recreation during his vacations, he hurried back to the modest laboratory which he had set up in a small room in his parents' home. There he concentrated his thoughts and energies on his own scientific methods and problems.

Perhaps many university students in the field of science today are not cognizant of what was required to become a scientist in the early days of Paul Ehrlich. When we talk about chemistry, physics, mathematics, and medical science, there is a tendency now to combine with these concepts thoughts of extraordinary test plants, complicated equipment, and laboratories equipped with the latest achievements of modern engineering technology. If we consider, for example, the field of nuclear science, then our minds visualize particle acceleration and atomic piles. If we discuss chemistry, we think in terms of scientific pilot plants for synthetic fuels, rubber, fibers, and plastics.

In the field of mathematics, we now are familiar with the giant computers capable of producing in a matter of minutes the solution of a complex mathematical problem which would take an expert mathematician weeks, months, and perhaps even years to work out. Our age of technology has developed automatic control equipment for modern production processes which operates more efficiently than man, and complex diagnostic and remedial equipment for the field of medicine. Wherever we look, we find the highly developed media which are governing science today.

There is a vast difference between the prospects that confront the student today and those that confronted Paul Ehrlich when he was studying in a university and spending his vacations in his own laboratory. The individual scientist in Ehrlich's day was hardly a part of a large scientific research organization. Nor was he necessarily a member of a team of scientists working in the same or similar fields. The scientist of Ehrlich's day was mostly on his own.

Contrary to the high degree of specialization which is characteristic today, a far-reaching and profound over-all knowledge was one of the most significant properties of the scientist of yesterday. And so it was of Paul Ehrlich. His success was largely attributable to his varied knowledge, which spread far beyond the medical field and into the branches of chemistry, physics, mathematics, and classical studies.

I should like here to touch briefly on my own experience, for when I began the study of chemistry in 1925, science was in a period of transition. Men such as Tamman, Szigmondy, Bodenstein, and Windaus, under whom I studied, were, with their genius, obtaining conclusive results by relatively simple means. But at the same time, the modern tools which now are indispensable in the fields of science were conceived and developed.

Still another change was taking place. By the time I had finished my studies and had entered professional life, it was accepted that scientific teamwork was necessary if new fields were to be conquered. Of course, Ehrlich cooperated with many of the outstanding scientists of his time, including such men as Koch and Behring, but this cooperation was more in the line of individual association than in the sense of teamwork as we know it today.

Now some people might come to the conclusion that, while the tools in chemistry, physics, and medicine have changed, there also is a corollary change in the basic requirements for the fiber out of which a good scientist is made. I am not of this opinion. On the contrary, I think that we have never needed the qualities of high character, professional competence, unselfishness, and devotion to searching for the truth more than we need them today, and for many reasons. The tools that are now available have not only opened broad new areas, hitherto unexplored, in the field of science, but have immeasurably increased the responsibilities of scientists.

We cannot even grasp, let alone fulfill, the scope of these responsibilities, unless we possess the qualities that I have enumerated. If we search for the truth, we cannot be blind to the fact that there are only two roads before us: the road that leads to a final disaster, and the other road that leads to an age in which science and technical skill are equaled by the moral and spiritual forces of mankind that will harness those powers that are now, for the first time, resting in our hands.

Some people might think, perhaps, that there is a vast gap between an observance of the Ehrlich Centennial and these meditations about our responsibilities of the day. I do not think so. If we honor great men of the past it is for the example they have set us; and their example exacts a pledge from us to live up to their ideals. What we need in our unprecedented world situation is guidance; and where could we find better guidance than in contemplating the life of such men as Paul Ehrlich and Behring, who were not only great scientists but who also count among the great humanitarians of our world? It is the combination of science and humanitarianism that gave these men their stature.

In presenting these busts on behalf of German scientists, of whom I am one by training, I feel there is a spiritual obligation to strive after the ideals of men such as Ehrlich and Behring and to take the example they set as a challenge. If we accept this challenge, and remain firm in our never-ending search for scientific truths and in our devotion to the humanitarian principles which guided Paul Ehrlich and Emil von Behring and, if thus the powers of our hearts equal those of our brains, then and only then shall we contribute to a better world.

INTRODUCTORY REMARKS

By E. Grunberg
Chemotherapy Laboratories, Hoffmann-La Roche, Inc., Nutley, N. J.

We have gathered here in order to commemorate the 100th return of the birthday of Paul Ehrlich. It is my privilege and honor to welcome you to this conference, in which we have tried to give you an outline, as complete as possible in the framework of a one-day conference, of Paul Ehrlich's work and its influence on experimental medicine. This influence has lasted to this day, and it will probably continue to have a stimulating effect in the future. Competent speakers, among them a few of Paul Ehrlich's co-workers, will address you in the course of the next hours and will tell you, much better than I can, about Ehrlich's achievements and the development of his scientific concepts. Let me here only remind you that Paul Ehrlich, together with Louis Pasteur, Robert Koch, Joseph Lister, and Emil von Behring, belongs to the group of outstanding scientists of the 19th century who have laid the foundations of modern medicine, particularly in the field of infectious diseases, either by discovering their causes or by showing new and successful ways of combating them.

The name of Emil von Behring, the discoverer of antitoxic sera and one of the most prominent figures in the field of practical immunology, reminds us also of the fact that Ehrlich, in an early phase of his work, was closely connected with von Behring. Moreover, both were contemporaries, and only a few days ago, on March 15th, the centennial of Emil von Behring's birthday also occurred. Let us include the memory of this great man in today's commemorations.

A glance at the program will show you the multitude of aspects of Ehrlich's scientific work which will be presented to you. The list is incomplete and there are many more fields in which his ingenious concepts have exerted their stimulating influence. We have felt justified in not exhausting either the representation of Ehrlich's scientific personality or the patience of you, the listeners, because what we are going to celebrate and commemorate is not only a scientist, but an idea. This idea, conceived early in his career and sustained through all his life, is the assumption of a direct chemical relationship of active substances to cells, the cells of the host as well as parasitic cells. Ehrlich's staining studies, carried out while he was still a student, the side-chain theory of immunity, and the chemoceptor theory of chemotherapy are all expressions and applications of the same principle: the specific affinity of chemical substances to specific cells or cell systems. Although we might be able today to express these affinities more accurately and refer to well-defined enzymes and enzyme systems, the principle recognized or intuitively anticipated by Ehrlich is still the same principle.

Before turning over the meeting to the speakers, I should like to extend a special welcome to Ehrlich's former co-workers who graciously consented to come here and talk to us: Doctor C. H. Browning and Doctor Hugo Bauer.

It is also my pleasure to welcome Doctor Heinz Krekeler, Ambassador to the United States of the German Federal Republic, who has joined us here in order to honor a man who was born in Germany and lived in Germany all his life, though the fruits of his work belong to mankind.

PAUL EHRLICH'S INFLUENCE ON CHEMISTRY AND BIOCHEMISTRY

By Hugo Bauer

Natonal Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md.

The concept of the importance of chemistry to the development of the biological sciences was slowly recognized at the end of the past century. The genius of Pasteur, who was a chemist, had brought about the most spectacular progress in applying chemical methods to medicine and to the study of human and animal diseases. To a certain extent, it was Paul Ehrlich who succeeded in realizing a union of chemistry and biology. "There is no single man," said Leonor Michaelis of Ehrlich, "who is able to appraise his work." The truth of this assertion is evident from the number of scientists who deal today with his researches.

In this review of Paul Ehrlich's influence on chemistry and biochemistry, I have made use of the writings of many authors.[1-5] Richard Willstätter, in his appraisal of Ehrlich's work, said: "As a chemist, P. Ehrlich is no other master's disciple. He was not carried by currents of his time or even influenced by them; through his intuition and the vigor of his personality new fields were opened to us. His work constitutes not only a new, fruitful method of pharmacology, at the same time it has great influence in bringing endless material of synthetic chemistry into biologically directed paths and opening up new and fruitful tasks to synthetic chemistry."

The astonishing diversity of Ehrlich's work can be comprehended when we realize that his pioneering mind was always in search of new unsolved problems. After having opened a new field of research, he left it to his successors to continue on his path, while he himself turned to new problems.

The sharpest critic of his work was Ehrlich himself. All possible objections he had himself and tested their value experimentally. "I could have been satisfied," Ehrlich said, "to relate only facts and to renounce discussion. But in my opinion, a progress of knowledge can be made only from the theoretical viewpoint, and therefore even a wrong theory may be more fruitful than crude empirical registration of facts."

Thus Ehrlich was conscious of the common experience that theories and hypotheses are always superseded, and that the value of new ideas is shown by their stimulating influence on future investigators.

Ehrlich himself in his chemical work made use almost exclusively of the test tube. He followed up each reaction to the point from which he could form a view of the course of the reaction. He then discussed his findings with a collaborator and a working project was set up.

There are three periods we can distinguish in Ehrlich's scientific work: (1) analytical studies in dyes; (2) studies on immunity; and (3) chemotherapy.

Chemical concepts pervade all these periods. We may say, if he liked chemistry, he loved the dyes. When I worked as a young assistant to him in

1909, he said: "I should have been a dye chemist." At that time Ehrlich was interested in the combination therapy of arsenicals with therapeutically active dyestuffs. The work with his beloved dyes is one of the guiding principles in the evolution of Ehrlich's researches.

Dyes were used before Ehrlich's time for staining microscopic preparations. However, the concept of the specific affinities of dyes was verified in the first place by Ehrlich's classification of dyes as basic and as acid.

To enumerate his achievements: the discovery of the mast cells, an acid fast stain for the tubercle bacillus, the selection of methylene blue for staining gonococci, the fixation by oxidation with benzoyl peroxide, the use of methylene blue for the staining of nerve tissues, and the staining of tissues in living animals, the so-called vital staining.

The use of dyes provided means of making visible the reducing and oxidizing properties of the tissues of living animals, as described in his *Sauerstoff-bedürfnis des Organismus*.[6] Ehrlich brought the living cell in contact with alizarine blue or indophenol, dyes whose reduction can be recognized by decolorization. The idea of measuring chemical energies by means of reversible processes was far ahead of his time. Here, we find the germ of his "side-chain theory," on which he later based his chemotherapeutic theories.

In the aspect of enzyme chemistry, we may assume that, in asphyxiated cells, the sulfhydryl groups of enzyme systems are liberated, that the hydrogen carriers (*dehydrogenases*) are in the reduced state and readily furnish hydrogen to reducible linkages.

The second period of Ehrlich's work started with the discovery of the diphtheria toxin by Behring.

The quantitative work on the problem of immunity was led into new tracks when Ehrlich introduced the test tube experiment into the study on toxin and antitoxin. He succeeded in showing that ricin is made innocuous by antiricin with exclusion of any assumption of the cell's activity.

The quantitative determination of the diphtheria serum could not have been achieved without applying methods derived from chemistry. Ehrlich was the first investigator to apply the concept of chemical reactions, particularly the concept of neutralization, to the problem of quantitative measurement of antigens and antibodies. "*Corpora non agunt nisi fixata*," and the fixing is owing to chemical activity.

The chemical aspects of the formation of antibodies were further developed by Landsteiner, Breinl and Haurowitz, Heidelberger, Pauling, and many others. The chemical character of serological specificity has become more and more manifest.

Landsteiner states that antibodies are now definitely recognized as modified globulins.

Pauling assumes that antibodies differ from normal serum globulin only in the way in which the two end parts of the globulin polypeptide chain are coiled. The coiling of the end parts is modified under the influence of an antigen and offers a great variety of configurations. These modern concepts appear as refinements and do not differ principally from the old side-chain theory.

The results of Ehrlich's investigations on tissue staining led to questions of pure chemistry.

His studies on the condensation of aromatic nitroso compounds with methylene derivatives, in collaboration with Franz Sachs, opened a fruitful area which was cultivated for many years by his successors. Many ketones and polyketones were made available by means of the new methods.

$$CH_3COCH_2COCH_3 \rightarrow CH_3{\cdot}CO{\cdot}CO{\cdot}CO{\cdot}CH_3$$

Acetylacetone *Triketopentane*

(Sachs and Barschall)[7]

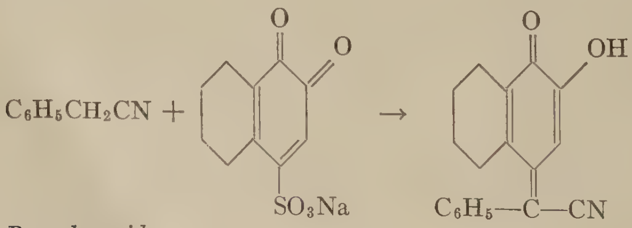
In collaboration with A. Einhorn, substituted derivatives of cocaine and azo dye derivatives were prepared.[8] This method of detecting reactive methylene groups was one of the tests used by Willstätter in the elucidation of the constitution of atropine and cocaine.

Another achievement of importance was the discovery by Ehrlich of reagents which couple with pyrrol and its derivatives. As early as 1883, Ehrlich had observed that diazotized sulfanilic acid couples with bilirubin. This diazo reaction of the urine was useful in the diagnosis of diseases such as typhoid fever. It has played an important role in all investigations on pyrrol derivatives, such as are found in the degradation of hemoglobin, of the bile dyes, and of chlorophyll. Most successfully this reaction was used by Hans Fischer in his studies on hemopyrrols.

Another valuable reagent is dimethylaminobanzaldehyde, used for the detection of physiologically important derivatives of pyrrol in pathological urines. Dimethylaminobenzaldehyde condenses with amino groups to give an orange colored derivative, and with active methylene groups to give a purple color.

One of the strangest condensation reactions carried out by Paul Ehrlich and A. Herter[9] was the coupling of o-naphthoquinonesulfonic acid with various materials. The reaction with aromatic amino compounds occurs, as found by M. Böniger, with the elimination of the sulfonic group and the replacement of it by an organic residue. A host of applications was made by Ehrlich, *e.g.*, the test for aniline which had been injected into animals, the substitution of acidic methylene groups, the conversion of alkaloids into dyes.

The reaction with methylene compounds was further investigated by F. Sachs and M. Craveri:[10]

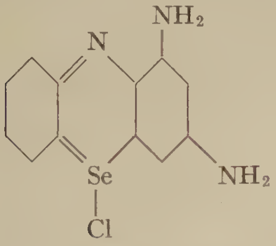


Benzylcyanide

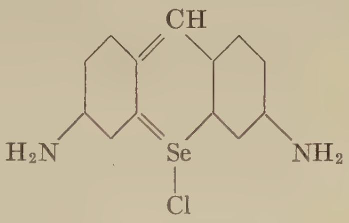
Recently this reaction was applied to the condensation of 1, 2-naphthoquinone sulfonic acid with substituted aminobenzamides and sulfonamides (N. Steiger).[11]

P. Ehrlich and F. Sachs[12] describe a delicate lecture demonstration concerning the preparation of triphenyl methane dyes. In this experiment, the Grignard method was made workable. By activating magnesium with ethyl bromide and ether, the heretofore unknown magnesium derivatives of halogenated amines were prepared. The bromomagnesium dimethylaniline thus obtained combined easily with Michler's ketone to form hexamethyltriaminotriphenylcarbinol (crystal violet). The authors demonstrated how the colorless triphenylcarbinol can be converted to orange, green, or purple compounds by successively introducing three dimethylamino groups.

For a short time, selenium compounds seemed to be of interest in the chemotherapy of cancer, according to Wassermann.[13] Upon Ehrlich's suggestion, H. Bauer synthesized o-aminoselenophenol, o-nitrophenylselenocyanide and o-aminophenylselenocyanide. By condensation of the latter selenocyanide with picrylchloride, 1, 3-dinitroselenazine was prepared. The 1, 3-diaminoselenazine, obtained by reduction of the dinitro compound, showed some action on trypanosomes, eliminating the blepharoplast, but was inactive in cancer. In the pyronine series, 3, 6-diaminoselenopyronine was prepared. It showed only weak action on trypanosomes.



1, 3-Diaminoselenazine[14]



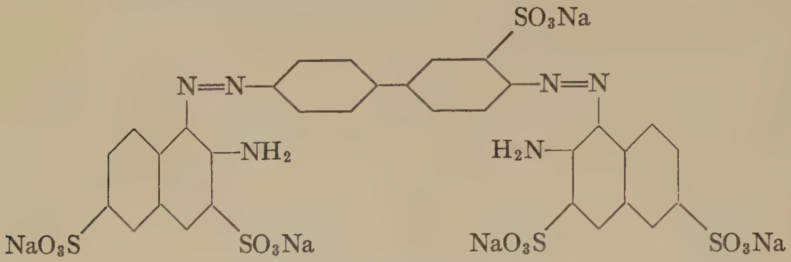
3, 6-Diaminoselenopyronine[15]

Ehrlich's influence on the dyestuffs industry is best illustrated by the following example. After the discovery of the affinity of methylene blue for nerve tissue, Ehrlich wanted to find the reason for this unique specificity. Ehrlich suggested to the Badische Anilin und Sodafabrik that they prepare the corresponding oxazine by condensation of nitrosodimethylaniline with m-hydroxydimethylaniline. The latter, not available then, had to be prepared from m-aminodimethylaniline applying the Griess method. His project was carried out, and the m-hydroxydimethylaniline proved to be a valuable intermediate for preparing new series of phthaleine dyes, the rhodamines, of Nile blue and other oxazine dyes, a memorable interaction of practice and theory, as H. Caro said.

Ehrlich's work in experimental chemotherapy was started in 1891, when he found that methylene blue stained malaria parasites very effectively. In collaboration with Guttmann, the dye was tried on malarial patients. Cases of tertian malaria were benefitted, but the results were not sufficiently impressive. However, this work played a guiding part in the development of the antimalarial drugs plasmochin and atebrin,[3] as we shall see later.

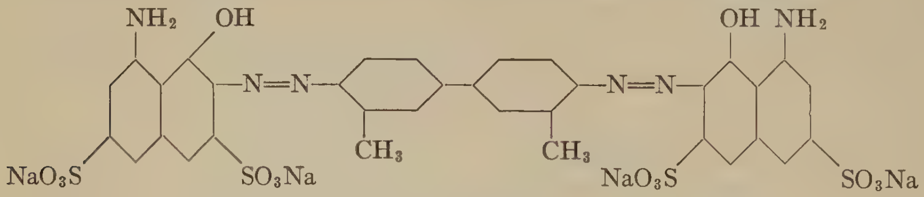
In collaboration with Shiga, Ehrlich started on the experimental chemotherapy of trypanosome infections (1904) largely because there was no known cure for sleeping sickness. After preliminary work on the benzopurpurine series of dyes, von Weinberg and Ullmann synthesized, upon Ehrlich's proposal, a tetrazo dye, trypan red.[16]

The dye was both curative and prophylactic for mice infected with the disease of *mal de caderas* (Trypanosoma equinum).[17] This was the first cure of an experimentally produced disease by administration of a synthetic organic substance of known chemical composition.[3]

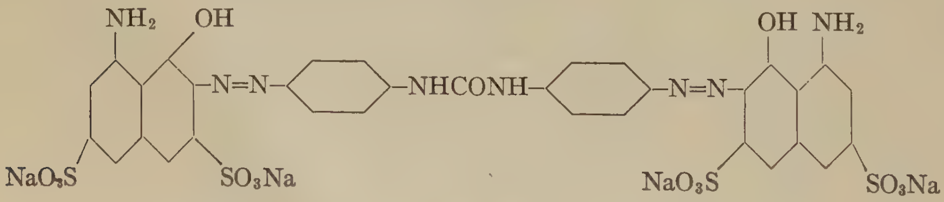


Trypan red

The discovery led to the investigation by F. Mesnil and M. Nicolle[18] of two other dyes of the same series, Trypan blue and Afridol violet. Trypan blue was used for the treatment of trypanosomiasis in cattle, but, since bright blue meat was unsalable,[3] the Bayer Co. attempted to find a colorless trypanocide.

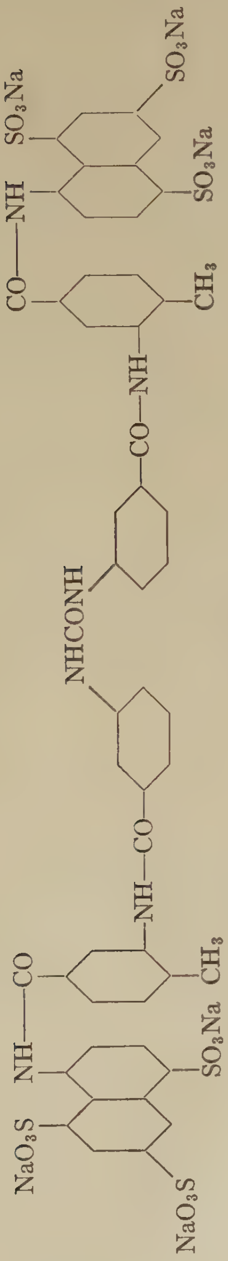


Trypan blue



Afridol violet

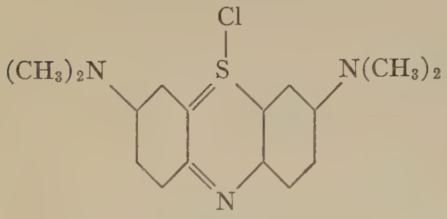
Afridol violet contained one urea linkage. Using this principle, the chemists of the Bayer Co. in Elberfeld, Heymann, Dressel, and Kothe, after preparing 2,000 compounds, developed Bayer 205 or Germanin (Suramin). Bayer 205 has proved to be an extraordinarily useful trypanocidal agent. Its chemotherapeutic index for mice is about 300, the highest for any compound known at that time. Its constitution was kept secret, but was finally revealed by E. Fourneau and his associates.[19]


SO₃Na
SO₃Na
SO₃Na
CO—NH
CH₃
NH—CO
NHCONH
NH—CO
CO—NH
CH₃
SO₃Na
NaO₃S
NaO₃S

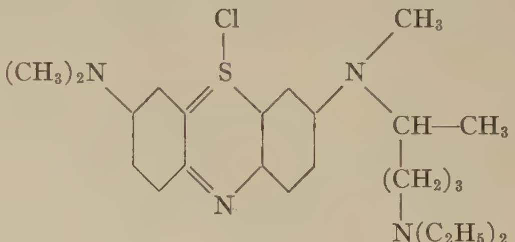
Germanin

An apparently insignificant change in the molecule of trypan blue, namely the omission of the two methyl groups, abolished trypanocidal activity. The same is the case with Bayer 205. No explanation can be given for this striking effect.

Other successful investigations originating from the work of Ehrlich were carried on in the antimalarial field. As already mentioned, Ehrlich, in 1891, found that methylene blue had some curative effect on malaria. The Bayer research chemists accordingly tried the effect of replacing the methyl groups attached to the aromatic amino groups of methylene blue with other alkyl groups. Next, they tried replacing the methyl groups with basic alkyl groups, producing compound II with the favorable chemotherapeutic index of 8.



Methylene blue

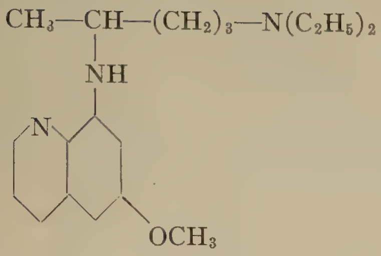


II

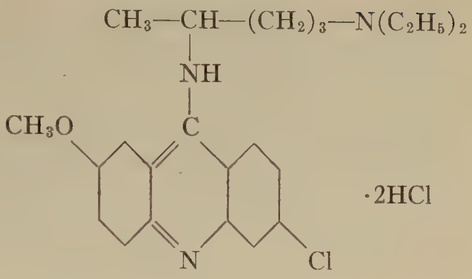
The work on methylene blue did not produce a compound of any practical value, but it indicated some relationship between antimalarial effect and chemical structure. The experience gained was applied to other ring systems, with the result that plasmochin, a derivative of 6-methoxy-8-aminoquinoline having a therapeutic index of 30, was produced in 1924 (Schulemann, Schönhöffer, and Wingler, 1932). Success in the quinoline series naturally led to an examination of similar substitutions in other heterocyclic nuclei, and led eventually to the acridine derivative known as atebrin or quinacrine (Mauss and Mietzsch, 1933; Kikuth, 1932).

Over 12,000 compounds, according to Schulemann, were tested before atebrin was found. Atebrin is well tolerated and its effectiveness regarding prophylaxis and treatment of malaria is about three times stronger than that of quinine. Tertian malaria is not cured by atebrin, neither by the newer drugs, chloroquine or paludrine.

Of the various halogen substituents, chlorine in position 6, is the best, iodine the least effective. Compounds lacking the halogen substituents showed only



Plasmochin, Pamaquine



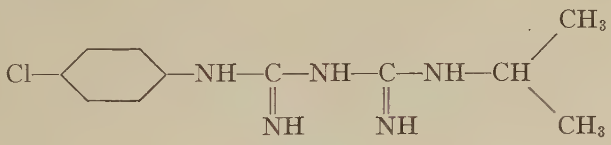
Atebrin, Quinacrine

weak activity. When the chlorine was replaced by the amine group, the effectiveness was completely abolished.

Substitution of position 6 by a nitro group depresses the specific action on malaria schizonts; however, there appears action against trypanosomes and streptococci. Systematic investigations on nitroaminoacridines were carried out by Schnitzer and Silberstein.[20]

Up to this point, all drugs which proved to be successful in the fight against malaria, the natural quinine alkaloids as well as the synthetic remedies, had, in common, a quinoline (or acridine) nucleus together with a basic side chain. The influence of the constitution of the side chain on therapeutic effectiveness was investigated by Mauss (1942), by Prelog (1943), and by their associates.

Atebrin has now been supplemented by paludrine, an antimalarial drug synthesized by chemists of the Imperial Chemical Industries of Great Britain in 1944 (Curd and Rose, 1946).



Paludrine

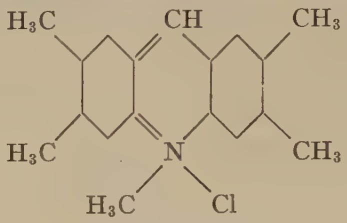
Paludrine is a biguanidine compound, the result of a search for antimalarial drugs among pyrimidine derivatives. Working on the theory—promoted by Schönhöffer for atebrin—that the antimalarial activity of pyrimidines may be associated with the possibility of tautomerism, Curd and Rose approached the biguanidines with the result that highly active derivatives were produced. Optimum antimalarial activity always required a terminal isopropyl group.

Paludrine is proving to be a most potent and nontoxic antimalarial drug. It is active against all three forms of malaria, and possesses the property, absent from other antimalarial drugs, of preventing the development of parasites in the pre-erythrocyte stage following primary infection (Fairley, 1946).

Among the rules of importance which became apparent in the course of the studies of dyes are the unfavorable influence of some groups like the methyl and nitro groups, the weakening influence of the sulfonic and carboxylic groups, and the favorable influence of chlorine.

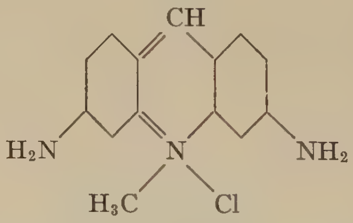
Thus, the therapeutic value of the rosaniline dyes is enhanced by the decrease of the number of methyl groups (however, note the exception in the case of germanin) and reaches a maximum with the introduction of chlorine. The chlorinated pararosaniline trypanosan (Ehrlich and Benda) was found to be the most effective of these dyes in trypanosomal infections.

The same unfavorable effect of methyl groups attached to the benzene ring was observed in the acridine series. Of the acridine derivatives, the acridinium yellow (F. Ullmann)[21] especially showed strong activity.



Acridinium yellow

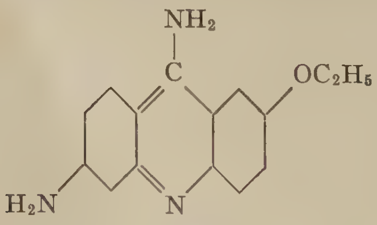
Based on his previous experience, Ehrlich induced L. Benda[22] to prepare the lower homolog of acridinium yellow, containing free amino-groups. In fact, this dye, having the same toxicity as acridinium yellow, was three times as effective against trypanosomes. This compound was named Trypaflavin. As a consequence, more dyes were developed, for instance cyanotrypaflavin and bis-trypaflavin.



Trypaflavin

Trypaflavin was of no practical importance in the treatment of trypanosomiasis. However, in 1917, C. H. Browning, who worked with Ehrlich, recognized the potency of trypaflavin or acriflavin as a wound disinfectant.

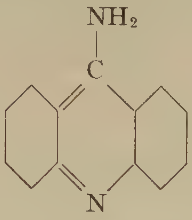
Further development of the acridine dyes led to the synthesis of "rivanol" by Roser and Jensch (1922), investigated by Morgenroth and Schnitzer.[23]



Rivanol

Rivanol is an antiseptic compound of little irritating properties and strongly bactericidal. Recently it was shown by A. Weizmann[24] that the addition of methyliodide or methylsulfate to form an acridinium salt increases the bactericidal activity.

The most important bacteriostatic agent of the acridine group is 9-aminoacridine, which was widely used as a wound antiseptic.



9-Aminoacridine

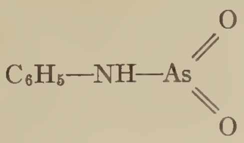
Early in Ehrlich's investigations the phenomenon of drug resistance was discovered. Ehrlich countered this phenomenon by combining drugs in order to attack the parasites from various points and thus created combination therapy. Drug resistant strains were valuable for the detection of new types of therapeutics and served as a "*cribrum therapeuticum*".

This drug resistance has given evidence for the direct action of drugs. From this background, the Interference Phenomenon of Browning and Gulbransen[25] was developed. The further investigation of the interference phenomenon by R. Schnitzer[20, 26] could explain some inconsistencies of Ehrlich's chemoceptor theory.

Ehrlich's pioneering work on chemotherapy was brought to a climax when he entered the field of arsenicals.

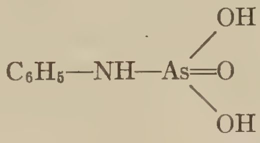
His idea that it was necessary to create institutes especially devoted to chemotherapy was realized in 1906, when the Georg Speyer-Haus in Frankfort-on-the-Main was built with the means of the foundation of Mrs. Franziska Speyer. Here Ehrlich created an organization, leading a group of scientists.

The work on arsenicals was started in 1902, when Ehrlich and Shiga[16] tried the action of atoxyl on trypanosomes. Atoxyl was the sodium salt of a compound obtained by Béchamp[27] in 1863 by heating aniline arsenate at 190 to 200° C. It was considered by him to be an anilide of arsenic acid according to one of the formulas:



$$C_6H_5\text{—}NH\text{—}As\begin{array}{l}/\!\!/\,O\\ \backslash\!\!\backslash\,O\end{array}$$

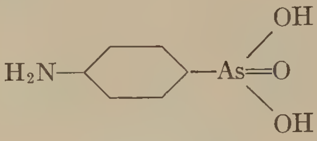
or



$$C_6H_5\text{—}NH\text{—}As\begin{array}{l}/\,OH\\ =O\\ \backslash\,OH\end{array}$$

However, working with an arsenic resistant strain of trypanosomes, Ehrlich and Shiga found no action. When, in 1905, Thomas and Breinl, using a different strain, detected some activity, Ehrlich's interest was awakened again. Atoxyl, introduced in 1906 by Robert Koch for the treatment of African sleeping sickness, was a dangerous drug because of its high toxicity and its detrimental effect on the optic nerve. Nobody thought ot the possibility that the action of a compound was a function of the chemical constitution. To have recognized this fundamental idea and to have it put into action is Ehrlich's own accomplishment.

Experimenting with atoxyl, Ehrlich made the surprising observation, that the compound reacted with nitrous acid to form a product which could be coupled to yield azo dyes. This fact was not in agreement with the assumed formula of atoxyl as an anilide of arsenic acid. Together with A. Bertheim,[28] the true constitution of atoxyl as the sodium salt of p-aminophenylarsonic acid or arsanilic acid was disclosed:

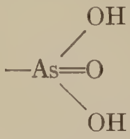


Arsanilic acid

The arsonic acid group Aso_3H_2, paralleling the reactions of the nitro group, is capable of a variety of chemical reactions, and just these transformations are of the greatest interest and practical value.

Germicidal activity cannot be determined in the test tube. Ehrlich demonstrated that certain arsonic acids do not act *in vitro*, but do so *in vivo*. He concluded that the arsonic acids had to be reduced in the body of the host from the pentavalent to the trivalent state to be rendered parasitocidal. In order to relieve the host of this labor, the reduction was carried out in the laboratory.

There are three stages of reduction of the arsonic acid group: the arsenoxide or arsinous group, the arseno group, and the arsine:



$-As(OH)_2{=}O$

arsonic acid

$-As{=}O$

arsinoxide

$-As{=}As-$

arseno

$-AsH_2$

arsine

The arsenoxides were the most active products, but of high toxicity, and were therefore not considered by Ehrlich to be of practical value. As we shall see, this view had to be corrected. The arsenobenzenes, analogs of the azo dyes and of yellow color, were the ones which absorbed Ehrlich's interest. They showed a favorable therapeutic index, as expressed in the formula $\frac{\text{Curative dose}}{\text{Tolerated dose}}$. The smaller the quotient, the better the index. Sometimes the reciprocal value is used.

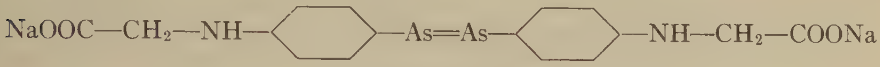
The arsines were difficult to prepare and did not find a practical application. Much of this work was carried out by R. Kahn.[29]

The reduction products of the arsonic acids were found to be very sensitive, especially to oxygen, and their preparation in a pure state was difficult. New methods had to be developed.

For the preparation of arsinoxides, sulfurous acid in the presence of a small amount of hydriodic acid as a catalyst was found to be a suitable procedure.

For the reduction of arsonic acids to arsenocompounds, sodium hydrosulfite, little used at that time, proved to be an excellent reagent.

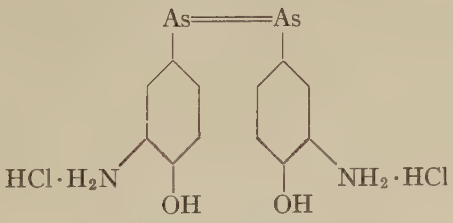
Special precautions had to be used to protect the arsenocompounds from oxidation by the oxygen of the air. The procedures were worked out in the preparation of arsenophenylglycine, a compound which had an almost ideal effectiveness in animals infected with trypanosomes, but was less satisfactory in humans.



Arsenophenylglycine

This compound is remarkable because it is active against trypanosome strains which are resistant to atoxyl, arsacetin, and salvarsan.

After the discovery of the *Spirochaeta pallida* as the parasite causing syphilis by Schaudinn (1905), Ehrlich directed his efforts towards the search for a spirochetocidal arsenic compound. The research, leading to numerous new arsonic acids and their reduction products, finally culminated in the preparation of 3,3-diamino-4,4′-dihydroxyarsenobenzene (May 1909), prepared by Ehrlich and Bertheim[30] and biologically tested by Hata.[31] It was designated Ehrlich-Hata 606, and the commercial name, in Germany, was salvarsan: in the United States arsphenamine.



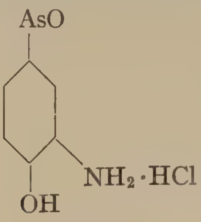
Salvarsan, Arsphenamine

Attempting to increase the stability of salvarsan solutions by addition of oxygen-absorbing agents, Ehrlich found that sodium formaldehyde sulfoxylate combined readily with salvarsan, forming a condensation product which is easily soluble in water with neutral reaction. It was introduced as Neosalvarsan.

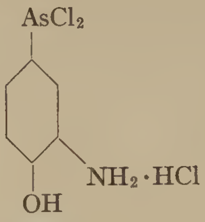
New derivatives were developed by P. Karrer.[32] Oxidation of arsanilic acid with persulfuric acid yielded p-nitrosophenylarsonic acid. This compound could be condensed with amino and aminoarsonic acids to form azocompounds. Other compounds obtained by Karrer are azides (diazoimides), dinitroso derivatives and arsenic containing phenazine dyes, iodoso- and iodophenylarsonic acid.

The combination of salvarsan with copper, silver, and gold salts gave compounds of complex nature.[33] Silver salvarsan was found to be of practical value.

Numerous arsenobenzenes were synthesized in the following years, mainly in the laboratories of the Farbwerke Höchst: sulfoxylsalvarsan, solusalvarsan, myosalvarsan; the latter is called in America sulfarsphenamine. The high toxicity of the arsenoxides caused Ehrlich to discard the use of the 3-amino-4-hydroxyphenylarsinoxide for therapeutic purposes. However, when prepared in a pure state as a hydrochloride, it was found to be useful and was introduced as Mapharsen or oxophenarsine.

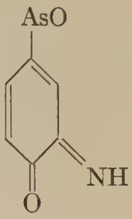


Mapharsen



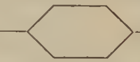
Dichlormapharsen

The o-aminophenol grouping makes the arsenic compounds highly oxidizable and probably promotes the formation of p-arsenoso-quinone-o-imine which is regarded as perhaps the ultimate toxic and therapeutically active compound in these series (Reiner & Leonard[34]).

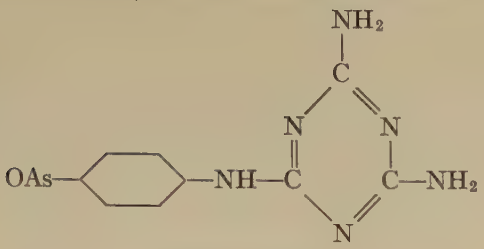


p-Arsenosoquinone-o-imine

Mapharsen has retained its position in the therapy of certain stages of syphilis. It is still listed in 14th Revision (1950) of the *United States Pharmacopeia*, while arsphenamine and neoarsphenamine have disappeared.

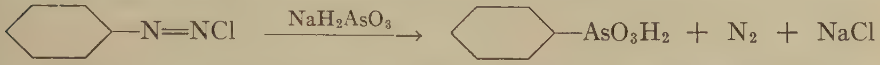
The introduction of acid groups into aromatic arsenicals generally diminishes or even abolishes the parasitocidal properties. A remarkable exception is the butyric acid derivative of phenylarsinoxide, OAs—  —CH_2—CH_2—CH_2—COOH, found by H. Eagle[35] to possess an excellent index for the *Trypanosoma equiperdum* infection of white mice. The compound is active against *Trypanosoma gambiense* in animals and humans. If administered to patients before the nervous system was attacked, 90 per cent could be cured in 1 to 2 weeks.

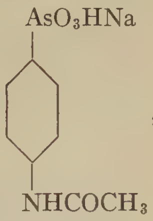
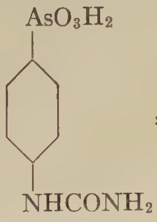
Another arsinoxide of remarkable trypanocidal activity was synthesized by E. A. H. Friedheim, who investigated the arsenic derivatives of 2,4,6-triamino-5-triazine.[36]

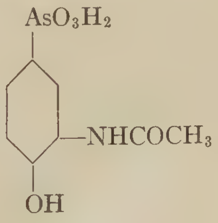


Melarsinoxide

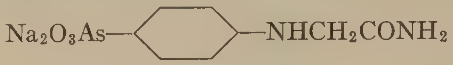
A new method of introducing arsenic into the aromatic nucleus was found by H. Bart,[37] who used the interaction of sodium arsenite with diazotized amines:



This reaction was further modified by E. Scheller,[38] who used arsenic trichloride halogenides for coupling, and by Ruddy, Starkey and Hartung,[39] who allowed arsenic trioxide to act upon the isolated borofluoride diazonium compounds. Arsonic acids were not closely investigated by Ehrlich, since his interest was occupied by the arsenobenzenes. However, a number of substituted arsonic acids were prepared and tested, the arsacetin , which was less toxic than atoxyl, the carbamino derivative , which was later known as "carbasone" and was used in amebic dysentery.

The 3-acetylamino-4-hydroxyphenylarsonic acid  was later investigated by Fourneau and introduced as Stovarsol for the therapy of syphilis.

Jacobs and Heidelberger[40] (1919) found the amide of the phenylglycine arsonic acid, its sodium salt called tryparsamide, to be a useful arsenical in the treatment of sleeping sickness and of syphilis of the nervous system. In contrast to the colloidal arsenobenzenes, tryparsamide behaves as a crystalloid and is able to penetrate the central nervous system.

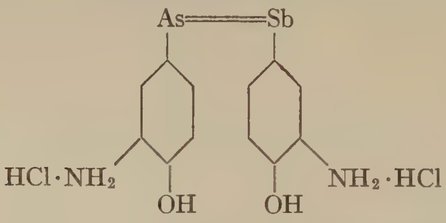


Tryparsamide

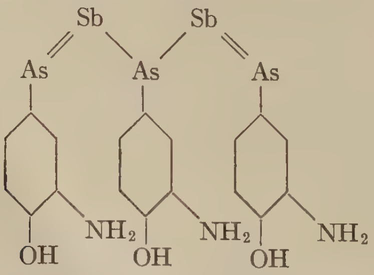
Arsonic acid derivatives of pyridine and their reduction products were synthesized by A. Binz and C. Räth in 1920–1933. None of these compounds was used clinically.

Today, over 10,000 organic arsenicals are known, according to Wagner-Jauregg.[4]

Mixed arsenostibino compounds were prepared by Ehrlich and Karrer,[41] by condensation of an arsine and a stibio-oxide or -chloride, analogous to the formation of azobenzene from nitrosobenzene and aniline:



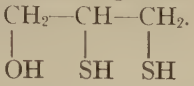
H. Schmidt[4,42] synthesized the following compound which reportedly was active in Bartonella infections not influenced by any other therapy:



Concerning the mechanism of action of organic arsenic compounds, it was found by Dustin,[43] Ludford[44] and Müller[45] that the cacodylates or atoxyl inhibit the mitosis of cells.

As already recognized by Ehrlich, arsenic in the trivalent form is the active principle. Of the organic arsenic compounds, only the arsinoxides are capable of killing the parasites *in vitro* in high dilutions.

Voegtlin and his collaborators provided the first practical demonstration of this theory.[3] It was shown that there is a lag period before arsonic acids and arsenobenzenes show their full activity. Voegtlin, Dyer, and Leonard,[46] and S. M. Rosenthal[47] studied the action of thiol compounds on arsenicals and found that, *e.g.*, thioglycol, thioacetic acid, reduced glutathion, or cysteine interfere with the action of the arsinoxides and arsenicals in general. So does the British antilewisite BAL, 2,3-Dithiopropanol:



$$\underset{\displaystyle OH}{\underset{|}{CH_2}}-\underset{\displaystyle SH}{\underset{|}{CH}}-\underset{\displaystyle SH}{\underset{|}{CH_2}}.$$

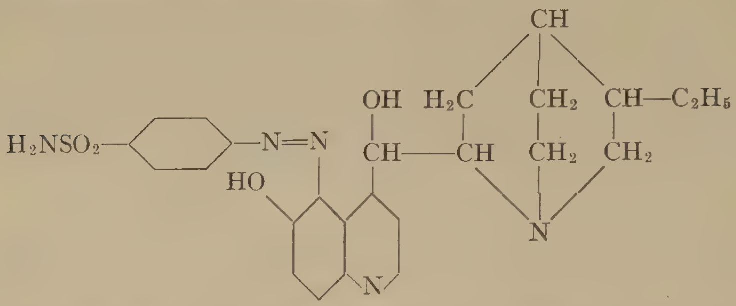
As T. Wagner-Jauregg[4] points out, trypanocidal action of arsinoxides is possibly caused by the inactivation of thiol compounds which function as activators in the carbohydrate metabolism. The thiol groups of the protozoans seem to be more vulnerable than those of the host. Only the arsenic sensitive protozoans take up arsenicals but not the arsenic resistant ones. Ehrlich's view of the direct action of the drug upon the parasite is confirmed by the newer investigations. The arsenoceptors may be identical with the thiol groups of the parasites. A different view is presented by A. Burger:[5] "The ideas of Voegtlin and his associates would explain inhibition of pathogens *in vivo* by arsenicals on the assumption that the parasites contain enzymes which are selectively poisoned, or for which the reversibility of the interaction with the arsenical drug has been greatly diminished. Actually, however, no enzyme fulfilling these requirements has been found yet in trypanosomes, spirochetes, or other organisms against which clinical therapy with arsenicals has long been used. Such universally distributed enzymes as pyruvate oxidase are rapidly deactivated by arsenicals, but Barron and Singer,[48] testing 18 different enzymes, could not observe significant differences in the degree of inhibition. Consequently, one cannot say that the number of sulfhydryl groups in enzymes is related to the toxicity of arsenicals for parasite cells. The permeability of cell walls for arsenic compounds seems to be a more important measure of the toxic manifestations of a drug, and, as shown by H. Eagle, the 'binding' of arsenicals by erythrocytes may serve as a model experiment for this mechanism."

Investigating the organotropy, Hogan and Eagle,[49] administered arsenicals whose toxic doses varied 500-fold. In spite of these differences, the concentration of arsenic in the tissues was nearly the same in three cases out of four.

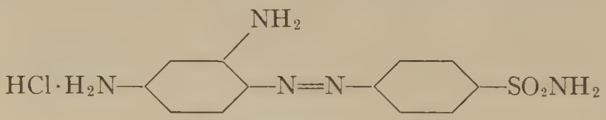
These data are consistent with the general thesis of Ehrlich that pharmacologic effects of arsenicals are determined by their combination with specific "arsenoceptor groups," whether in the tissues of the host (organotropism) or in the invading organism (parasitotropism).

No significant progress in the treatment of bacterial infections had been made. The study of modified antimalarial alkaloids of the cinchona group by Morgenroth and Levy[50] produced the ethyl ether of dihydrocupreine which under the name of "Optochin" was used in the therapy of pneumonia for a short time.

Dyes remained a major guiding principle of research in chemotherapy. A coupling product of diazotized p-aminobenzene sulfonamide with dihydrocupreine was tested by Heidelberger and Jacobs[51] in 1919:

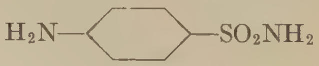


However, this dye did not show high antibacterial activity. The dyes which were the forerunners of sulfanilamide were synthesized by Mietzsch and Klarer and tested by Domagk in the Elberfeld Laboratories of the I. G. Farbenindustrie (1935). The introduction of the sulfonamide group —SO_2NH_2 derives from the experience in dyestuff chemistry. It had been known that acid wool dyestuffs containing this group were particularly fast to laundering, bleaching, and light, a criterion of strong affinity for protein molecules.[5] This affinity also extended to bacterial proteins, and in the course of preparing sulfonamide azo dyes, the historic drug Prontosil was discovered:



Prontosil

Tréfouel, Mme. Tréfouel, Nitti, and Bovet[52] recognized the sulfonamide moiety to be the active part of the Prontosil molecule. Consequently sulfanilamide was prepared and found to be highly potent.



Sulfanilamide

Ehrlich's achievements were recognized as the work of a genius even in his lifetime. On the hundredth anniversary of his birthday, we still feel the influence of his overpowering scientific personality. His work in chemistry alone, only a part of his great achievements, assures him of a high place among the really great explorers in the realm of science.

Bibliography

1. Ehrlich, P. 1914. Festschrift zum 60. Geburtstage Fischer. Jena, Germany.
2. Zinsser, H. 1939. Immunity. MacMillan. New York.
3. Work, T. S. & E. Work. 1948. The Basis of Chemotherapy. Interscience. New York.
4. Wagner-Jauregg, T. 1949. Therapeutische Chemie. Huber. Bern, Switzerland.
5. Burger, A. 1951. Medicinal Chemistry. Interscience. New York.
6. Ehrlich, P. 1885. Das Sauerstoffbedürfnis des Organismus. Berlin, Germany.

7. Sachs, F. & H. Barschall. 1901. Ber. **34:** 3047.
8. Ehrlich, P. & A. Einhorn. 1894. Ber. **27:** 1870.
9. Ehrlich, P. & A. Herter. 1904. Z. physiol. Chem. **41:** 379.
10. Sachs, F. & M. Craveri. 1905. Ber. **38:** 3685.
11. Steiger, N. 1950. U. S. Patents, (to Hoffman-La Roche) 2,551,647 & 2,554,543.
12. Ehrlich, P. & F. Sachs. 1903. Ber. **36:** 4296.
13. Wassermann, A. v. & D. v. Hansemann. 1912. Berlin, klin. Wochschr. **49:** 4.
14. Bauer, H. 1914. Ber. **47:** 1873.
15. Ehrlich, P. & H. Bauer. 1915. Ber. **48:** 502.
16. Ehrlich, P. & K. Shiga. 1904. Berlin. klin. Wochschr. **41:** 329, 362.
17. Ehrlich, P. 1907. Berlin. klin. Wochschr. **44:** 9–12.
18. Nicolle, M. & F. Mesnil. 1906. Ann. inst. Pasteur. **20:** 417, 513.
19. Fourneau, E., J. Tréfouel, Mme. Tréfouel & J. Vallée. 1924. Compt. rend. **178:** 675.
20. Schnitzer, R. & W. Silberstein. 1926. Z. Immunitätsforsch. **49:** 387.
21. Ullman, F. & A. Marić. 1901. Berl. **34:** 4307.
22. Benda, L. 1912. Ber. **45:** 1787.
23. Morgenroth, J., R. Schnitzer & E. Rosenberg. 1921. Deut. med. Wochschr. **47:** 317.
24. Weizmann, A. 1947. J. Am. Chem. Soc. **69:** 1224.
25. Browning, C. H. & R. Gulbransen. 1922. J. Path. Bact. **25:** 395.
26. Schnitzer, R. 1926. Z. Immunitätsforsch. **47:** 116.
27. Béchamp, A. 1863. Compt. rend. **56:** 1172.
28. Ehrlich, P. & A. Bertheim. 1907. Ber. **40:** 3292.
29. Kahn, R. 1912. Chem. Ztg. **36:** 1099; 1912. Z. angew. Chem. **25:** 1995.
30. Ehrlich, P. & A. Bertheim. 1912. Ber. **45:** 756.
31. Ehrlich, P. & S. Hata. 1910. Die experimentelle Chemotherapie der Spirillosen. Berlin, Germany.
32. Karrer, P. 1912. Ber. **45:** 2065, 2395; 1913. *Ibid.* **46:** 249; 1914. *Ibid.* **47:** 96, 1781, 2275.
33. Ehrlich, P. & P. Karrer. 1915. Ber. **48:** 1634.
34. Reiner, L. & C. S. Leonard. 1930. Proc. Soc. Exptl. Biol. Med. **27:** 788; 1932. *Ibid.* **29:** 946, 951.
35. Eagle, H. 1944. U. S. Pub. Health Repts. **59:** 765; 1946. *Ibid.* **61:** 1019; 1945. Science. **101:** 69.
36. Friedheim, E. A. H. 1944. J. Am. Chem. Soc. **66:** 1775.
37. Bart, H. 1922. Ann. **429:** 55, 103, 113.
38. Scheller, E. 1926. German Pat. 522,892.
39. Ruddy, A. W., E. B. Starkey & W. H. Hartung. 1942. J. Am. Chem. Soc. **64:** 828.
40. Jacobs, W. A. & M. Heidelberger. 1919. J. Am. Chem. Soc. **41:** 1822; 1919. J. Exptl. Med. **30:** 441.
41. Ehrlich, P. & P. Karrer. 1913. Ber. **46:** 3564.
42. Schmidt, H. & F. M. Peter. 1938. Advances in the Therapeutics of Antimony. Leipzig, Germany.
43. Dustin, A-P. 1933. Bull. acad. roy. méd. Belg. **13:** 585. 1934. *Ibid.* **14:** 487.
44. Ludford, R. J. 1936. Arch. exptl. Zellforsch. Gewebezücht. **18:** 411.
45. Müller, H. H. 1946. Naturwissenschaften. **33:** 253.
46. Voegtlin, C., H. A. Dyer & C. S. Leonard. 1923. U. S. Pub. Health Repts. **38:** 1882; C. Voegtlin, S. M. Rosenthal & J. M. Johnson. 1931. *Ibid.* **46:** 339.
47. Rosenthal, S. M. & C. Voegtlin. 1930. J. Pharmacol. Exptl. Therap. **39:** 347; S. M. Rosenthal. 1932. U. S. Pub. Health Repts. **47:** 241; 1942. J. Pharmacol. Exptl. Therap. **76:** 358.
48. Barron, E. S. G. & T. P. Singer. 1943. Science. **97:** 356.
49. Hogan, R. B. & H. Eagle. 1944. J. Pharmocal. Exptl. Therap. **80:** 93.
50. Morgenroth, J. & R. Levy. 1911. Berlin. klin. Wochschr. **48:** 1560, 1979.
51. Heidelberger, M. & W. A. Jacobs. 1919. J. Am. Chem. Soc. **41:** 213.
52. Tréfouel, J., Mme. Tréfouel, F. Nitti & D. Bovet. 1937. Presse méd. **45:** 839; 1937. Ann. inst. Pasteur. **58:** 30.

EHRLICH'S SIDE-CHAIN THEORY IN THE LIGHT OF PRESENT IMMUNOLOGY

By Ernest Witebsky

Professor of Bacteriology and Immunology, University of Buffalo School of Medicine; Bacteriologist, Buffalo General Hospital, Buffalo, N. Y.

It is indeed a special privilege and honor for me to be invited by your committee to discuss today, on Paul Ehrlich's 100th birthday, one of his most important and controversial contributions to science, the side-chain theory. My personal contact with Ehrlich's famous Institute in Frankfurt did not take place until a few years after Ehrlich's death, when Hans Sachs, Ehrlich's associate for 20 years and one of his most faithful students, introduced me to laboratory work. Ehrlich's powerful influence made itself felt even later, when I had the opportunity to work under Sachs in Heidelberg for a period of over eight years. Doctor Schnitzer is in a similar situation, having been associated with Julius Morgenroth, another important co-worker of Ehrlich's. We constitute a second generation of Ehrlich's disciples, so to speak. It is, therefore, a particular challenge to me to examine the significance of the side-chain theory as seen today in 1954, 57 years after Ehrlich's first communication on the topic.[1]

The Side-chain Theory

The concept of the side-chain theory can actually be divided into two major parts. The first part of the theory postulates a stereochemical basis for antigen-antibody combinations. The second part is concerned with an idea explaining the process of antibody formation.

In order to do justice to Ehrlich's theory, it might be well to understand just how the theory came about. The theory actually constitutes the dream of a genius, envisioned at the end of the last century, when very little was known about protein chemistry. The basis of this dream might have been Kekule's revolutionary concept of the chemical constitution of the benzene ring, whose reactions often depend upon the presence of small chemical radicals, or side-chains, connected to the six different carbon atoms. So Ehrlich thought of living matter as consisting of a central group reacting with its surroundings through the intermediary of definite specific units of unknown chemical constitution. Analogous to the side chains of the benzene ring, Ehrlich attributed to the side chains or *receptors* of the nucleus the function of reacting with the foodstuffs necessary to maintain life. The central nucleus would then be the actual basis of life. The side chains serve to anchor nutrient material and to make it assimilable by the central group. These receptors were referred to by Ehrlich, because of their physiological function, as *nutriceptors*. There was no way for nutrient substances to combine directly with the central nucleus in the absence of suitable receptors. The union of the nutrient material and the receptors of the cell was conceived on a clearly stereochemical basis, but in the absence of knowledge of the chemical constitution of these side chains, the term *receptor* was introduced as a symbol, lending itself readily to the schematic illustration of Ehrlich's concept.

Foreign material introduced into the circulation, avoiding the enzymes of the intestinal tract, cannot be used for direct assimilation in the synthesis of the individual's own protoplasm. Its fate would depend upon whether suitable receptors would be found in the animal body capable of direct chemical combination with the material injected. However, these foreign substances, which are not nutrients, can act upon the protoplasm in two different ways. If they are toxic, and a sufficient amount of the toxic material is anchored to the central nucleus through the intermediary of the side chains, the cell will be poisoned and will die. The combination of the nutriceptor with *non*toxic material will damage the central nucleus which needs the side chains for nutrition. According to Weigert's law, living cells would then not only replace the inactivated receptors but would overproduce them, and such a surplus of receptors would finally be cast off and secreted into the tissue fluids. These receptors present in the circulation were identified by Ehrlich as *antibodies*. Specifically that part of the cell receptor which would combine with the foreign material, or antigen as it was called, was referred to as the *haptophore* group, a symbol again to denote a chemical unit which would combine with the corresponding haptophore group of the antigen.

Three different types of receptors were postulated by Ehrlich, as illustrated in FIGURE 1.

A receptor of the *first order* was envisioned as containing just one single haptophore group. When secreted into the blood stream, as a consequence of antigenic stimulation, these receptors would combine directly with the antigen in question, usually a toxin. Such a toxin-antitoxin combination would lead to neutralization, but not necessarily to any visible precipitation.

A receptor of the *second order* was considered to be involved in antigen-antibody combinations which would produce a visible change, such as agglutination or precipitation. Receptors of the second order would have, according to Ehrlich, in addition to the haptophore group, a second group called the *ergophore* group, responsible for the macroscopic visible end-product of this type of antigen-antibody reaction.

A receptor of the *third order* was thought of by Ehrlich to be endowed with two haptophore groups, the one combining with food particles or antigenic material as the case may be, and the other with complement. This type of receptor, when secreted into the circulation, would appear as an antibody called *amboceptor*, acting as a bridge between complement and the cellular antigen.

I remember Sachs recounting how visitors used to come to Ehrlich's Institute wondering whether they could see the cell receptors under the microscope. Such questions always amused Ehrlich's collaborators and also drove them to despair because the diagrammatic drawings were obviously meant to symbolize chemical groupings of unknown composition. Emphasis was placed on stereochemical combination in definite proportions rather than on adsorption phenomena as viewed by Bordet and his school. Bordet,[2] in contrast to Ehrlich,

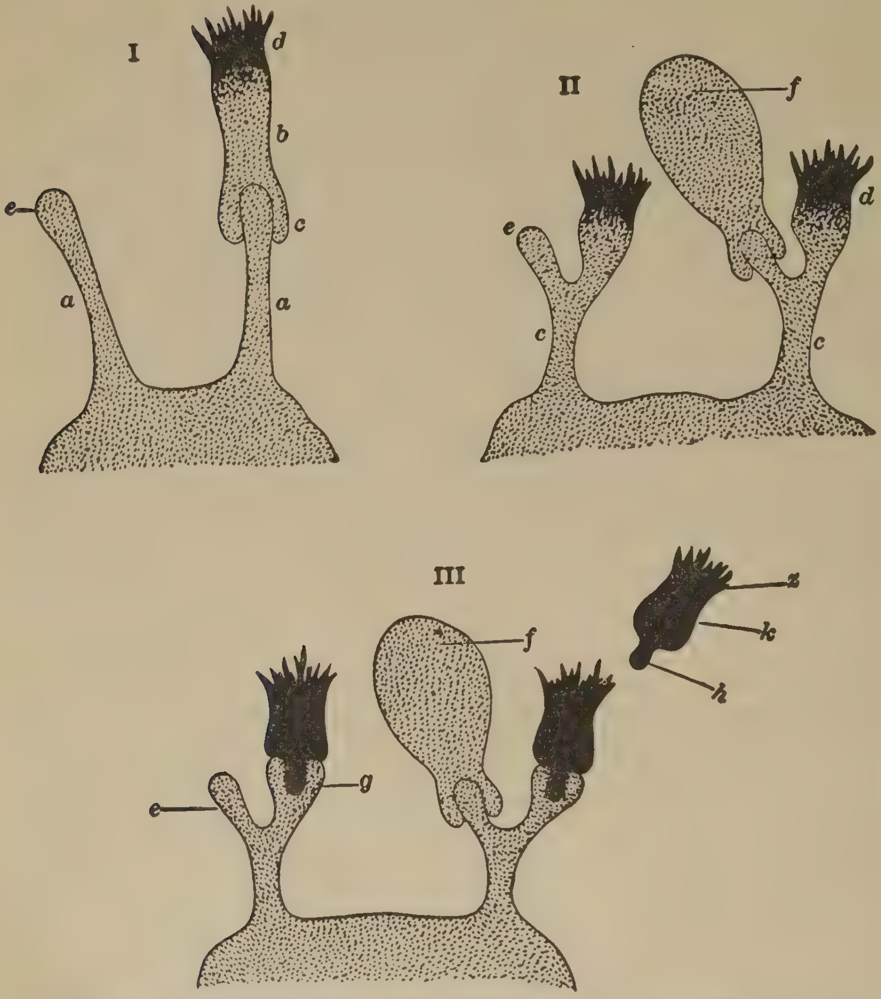


FIGURE 1. The various types of receptors according to Ehrlich. (I) Receptors of the first order. Here *a* is the receptor, with *e* its haptophore group; *b* is a toxin or enzyme, with *c* its haptophore group and *d* its toxophore group. (II) Receptors of the second order. Here *c* is the receptor, with *e* its haptophore group and *d* its ergophore group; *f* is a food molecule or antigen. (III) Receptors of the third order. Here *e* is the antigen-combining haptophore group and *g* is the complement-combining (complementophilic) haptophore group of the receptor (amboceptor); *k* is complement, with its haptophore group *h* and its ergophore group *z*. (From P. Ehrlich. 1901. Schlussbetrachtungen; Erkrankungen des Blutes; Nothnagel's Specielle Pathologie und Therapie, Vol. VIII. Vienna.)

tried to explain antigen-antibody reactions on a colloidal basis, and this difference of opinion remained one of the major items of dispute in the polemic between Ehrlich and Bordet, a discussion which proved so fruitful to science.

Ehrlich apparently felt antigens to be limited to a single valence and did not concern himself with any details of the mechanism of the visible reactions such as precipitation or agglutination, being more interested in the specificity of the primary antigen-antibody union. Bordet, on the other hand, apparently considered the antigen to be multivalent in the sense that it became coated with antibody molecules, but could not possibly explain by his theory the high degree of specificity characteristic of these reactions.

A certain experiment carried out by Sachs[3] in Ehrlich's Institute as far back as 50 years ago illustrates vividly what was doubtless in the mind of the originator of the side-chain theory. Sachs studied toxic substances (arachnolysin) extracted from a certain type of spider. These extracts were toxic for the red blood cells of some mammalian species and not for others. For instance, the red blood cells of the rabbit were quickly dissolved by the spider extract, whereas the red blood cells of the guinea pig remained unaffected. Investigations by Sachs with the shadows or stromata of red blood cells proved the existence of a substance in the stromata of the rabbit blood cells which was absent in the stromata of the guinea-pig blood cells. Spider lysin when mixed with rabbit blood stromata lost the ability to dissolve rabbit red cells. Mixed with guinea-pig stromata the spider lysin was not affected and still dissolved rabbit red blood cells. It always seemed to me that this simple experiment, carried out in Ehrlich's Institute such a long time ago, constituted compelling support for the actual existence of receptors. It showed how certain cells might be susceptible to different kinds of toxin, in contrast to other cells which are resistant. Furthermore, there is a close connection between susceptibility of cells to toxin and their power to combine with it. The specific combination of a toxin molecule and a susceptible cell by means of the corresponding haptophore group was compared with the action of an incorrectly constructed lightning rod directing the bolt of lightning into the heart of the structure. In contrast, freely circulating receptors were likened to correctly built lightning rods, deflecting the deadly effect of the bolt.

Diversity of Antibody Functions

The end of the last century undoubtedly was a time of explosive development of bacteriology and immunology. Within a short period of time, the basic types of antigen-antibody reactions were discovered, such as precipitation, agglutination, lysis, and phagocytosis. Each phenomenon appeared to be strikingly different, and Ehrlich attributed these various phenomena to different types of antibody structures. Therefore precipitins, agglutinins, lysins, *etc.*, were assumed to constitute independent factors of antiserum. However, there is no experimental evidence for the actual existence of these varieties of antibodies, and the so-called unitarian concept of antigen-antibody reactions has been generally accepted. The unitarian concept does not fail to recognize the basic differences in phenomena such as agglutination, lysis, and phagocytosis. The same antibody elicited by one antigen can produce different manifestations, it is believed, depending upon the physiochemical conditions of the antigen and on the environmental conditions. Precipitation would occur if the antigen is in solution; agglutination when the antigen is found on a cell, such as a bacterium or red-blood cell; lysis would occur in the presence of complement; phagocytosis in the presence of leukocytes or other phagocytic cells; and anaphylaxis in experimental animals. These diverse manifestations of antigen-antibody reactions can very well be explained as attributable to one and the same antibody function. But difficulties certainly have arisen when attempts have been made to explain allergic phenomena as being caused by this type of precipitating antibody. I believe that quite a few investigators stopped at that point, as I did, and still refuse to accept the belief that even the Prausnitz-Küstner antibody would be identical with a precipitating antibody.

An outstanding characteristic of antibodies lies in their specificity inasmuch as they are directed only against the antigen which caused their production and not against others. Ehrlich liked to use Emil Fischer's famous comparison of the fitting of a key into a keyhole to characterize this specific relationship.[4] The corresponding surfaces of the two molecules were considered to have complementary shapes, and thus to fit together and provide a stereochemical basis for their union.

Buchner believed one and the same agent present in blood serum to be responsible for agglutination of a variety of unrelated cells, such as blood cells and bacterial cells. Ehrlich, on the other hand, believed in the occurrence of a multiplicity of antibody molecules of different specificity, which caused agglutination of the various cellular elements. From the point of view of the specificity of antibody functions, Ehrlich and Morgenroth introduced the method of elective absorption,[5] by which they proved the existence of specific and independent antibodies directed against the different cells under investigation. The agglutinin-absorption technic constitutes a great and basic contribution to the understanding of the specific relationship between antigen and antibody. The classical studies of Ehrlich and Morgenroth on the receptor apparatus of red blood cells include their successful attempts to produce isoantibodies,[6] which are defined as antibodies directed against antigens occurring within the same species. Ehrlich and Morgenroth immunized a large number of goats and produced several isolytic sera, each of which acted on the blood cells of some goats but not on those of others. In this way, they could detect the existence of blood groups in this species. These experiments were carried out at about the same time as Landsteiner's basic experiments on the human blood groups.[7]

Investigations on red blood cells led Ehrlich to the belief that such cells must possess a great number of receptors of different types. According to Ehrlich's concept, the specificity of antigen-antibody reaction was limited to the interaction between an antigenic unit of the cell and its corresponding antibody. Assuming the existence of so many receptors on one cell, it was no surprise to Ehrlich that one kind of cell could elicit the formation of a variety of antibodies, each one directed against different receptor constituents of such cells.

The Horror Autotoxicus *Concept*

With experiences gained in studying the wealth of receptors on a single cell, Ehrlich conceived another ingenious idea on realizing that the injection of an individual's own red blood cells would never produce antibodies. Ehrlich coined the term *horror autotoxicus*,[8] a biological concept of great importance. No living organism would be capable of producing—or would *dare* to produce, if you wish—an antibody against constituents of its own body, for this would be incompatible with life. On the basis of the side-chain theory, the concept of *horror autotoxicus* seems logical, indeed. The principle of *horror autotoxicus* is a rather burning topic in the current literature, and constitutes a challenge for continued experimental study, testifying to Ehrlich's characteristically broad and far-sighted vision of biology.

Stimulated by the discovery of the Rh factor, the field of isoimmunization is going through a very productive period of investigative work. A great number of new blood factors has been discovered in human blood, the importance of which, in the practice of blood transfusion and in the field of hemolytic anemia of the newborn, cannot be overestimated. But the fundamental basis of all studies of isoimmunization is the operating of Ehrlich's law of *horror autotoxicus*. The tremendous mosaic of blood factors, appearing in a more or less characteristic pattern for each individual, never allows antibody formation against any of the factors present in the same individual. Isoantibodies are formed, either by transfusion or by pregnancy, only against those cell factors which are absent in the patient's own body. The validity of the law of *horror autotoxicus* certainly should be evident to everyone interested in the field of blood transfusion and blood disease. Autoantibodies—namely, antibodies directed against the receptors of the same individual—are not formed.

The study of the pathogenesis of the hemolytic disease of the newborn is especially exciting in the light of Ehrlich's point of view. Let us take the classical example of an Rh-negative mother who has produced an Rh antibody, stimulated by an Rh-positive baby. The mother is slowly but steadily infused with the baby's red blood cells containing the Rh factor, stimulating formation of Rh antibodies. As far as the mother is concerned, we are dealing with a clear case of isoimmunization, but because of the intricate permeability of the placenta, the Rh antibody formed in the mother passes through the placenta into the baby's circulation, acting there as an autoantibody on the baby's blood cells—certainly an amazing chain of events. The baby suffers blood destruction because there is a receptor on his cells, the Rh factor, which unites with the Rh antibody obtained from the mother. This situation constitutes a classical example of the two basic types of immunization recognized by Ehrlich on the basis of his receptor and side-chain theory. In the case of the mother, antibodies were produced by her own antibody-producing tissue, a process called by Ehrlich *active* immunization. In contrast, the baby, who had nothing to do with antibody production itself, but who received the antibodies from the mother, has been *passively* immunized. Thus, the cause of hemolytic disease of the newborn is clear. An antibody directed against constituents of the baby's own body is acquired in the form of passive transfer. The antibody doing its destructive work can be recognized on the baby's cells in different ways, the most widely used being the technic developed by Coombs. This technic demonstrates the attachment of the antibody globulin to the baby's cells, and will be further discussed below.

There is a second type of acquired hemolytic anemia, found mainly in adults. This serious disease frequently is a sequel to other diseases such as malignancies. Sometimes no apparent underlying cause can be recognized, in which case the disease is referred to as idiopathic. Antibodies can, on occasion, be demonstrated in the blood serum of the patient, and the Coombs' test with the patient's cells is usually positive. It has been postulated that acquired hemolytic anemia of the adult is an expression of an autoantibody formation and the experimental findings just mentioned are considered proof of the correctness of this explanation of the disease. In studying cases of acquired hemolytic anemia, one cannot help but be impressed by the parallel laboratory findings for acquired hemolytic anemia in the adult and the hemolytic disease of the newborn. At the same time, it seems to me unwise to overlook the fact that, in the case of hemolytic anemia of the baby, the origin and mode of action of antibody formation is understood clearly and beyond doubt. This mode of action is a passive form of immunization. In contrast, acquired hemolytic anemia of the adult would represent an example of active immunization against constituents of the patient's own body leading to autoantibody formation which could operate at body temperature. Such an autoantibody formation would certainly be in sharp contrast to the existing experimental knowledge about isoantibody formation and the law of *horror autotoxicus* which governs it. Both the nature of the antigens and the origin of antibodies involved in autosenzitization are still today a complete mystery. For these reasons, and for these reasons only, it would seem to me premature to close the books on the pathogenesis of acquired hemolytic anemia of the adult without further attempts to analyze it. A search should be also directed to find agents other than autoantibodies which could bring about the alteration of blood cells manifested in acquired hemolytic anemia of the adult.

The Antibody Spectrum

As stated before, Ehrlich assumed the existence of three different types of antibodies, according to the manifestations which would result from the combination of each of those types of antibodies with the homologous antigen. For many years, this concept of multiple antibody structures was rejected by many investigators, especially by firm believers in the unitarian concept of antigen-antibody reactions. Indeed, until recently, little could be said against this school of thought. But let us look at the record as it stands in the year 1954. Levine and Stetson[9] observed their first case of Rh isoimmunization in an Rh-negative mother by demonstrating an agglutinating antibody against the baby's and father's cells using saline solution as a diluent. Levine's theory for a while encountered considerable difficulties inasmuch as the majority of Rh-negative mothers who had given birth to an erythroblastotic child failed to contain Rh isoagglutinins demonstrable by the technics used at that time. Inability to demonstrate an Rh agglutinin in these cases was based upon the presence of an Rh antibody which though uniting with Rh-positive cells did not produce visible agglutination, as shown by Diamond,[10] Wiener,[11] and Race.[12] Antibodies of this kind were called by Race[12] *incomplete agglutinins*, in contrast to the *complete agglutinins* which produce visible agglutination.

We are dealing here, then, with an antibody of the type which, in terms of Ehrlich's ideas, would have been of the *first order*, missing the ergophore group. What methods are available to demonstrate the presence of such an antibody?

There are four such methods:

(1) Red blood cells possessing the Rh receptor are mixed with an antiserum containing *incomplete* Rh antibodies, which unite with the Rh receptor and cover it, so to speak. If these cells then are exposed to a *complete* Rh antibody, there will be no agglutination. The first antiserum apparently blocked the action of the second one by combining with the antigenic site. One could compare such a situation to a keyhole which is occupied by a key whose handle had been broken. An attempt to open the lock with a second key would be futile. Actually, similar observations on different examples have been made over a period of many years, and somewhat loosely defined terms, such as *agglutinoid* and *complementoid* were coined. These terms now seem to be both clearer and more realistic.

(2) The second method replaces the diluent, saline solution, by a protein medium containing undiluted normal human serum or serum albumin.[13] This change of diluent, in many cases, somehow transforms the *incomplete* Rh antibody action into a *complete* one, resulting in visible agglutination.

(3) The third method is based on the understanding that an Rh antibody, like many other antibodies, is associated with the gamma globulin fraction of the serum. This gamma globulin would then become attached to the sites of the cell containing the Rh receptors, without causing visible agglutination. The addition of an antiserum against human gamma globulin, obtained by immunization of rabbits or goats with that material, will therefore add a second antibody to cells sensitized with the *incomplete* Rh antibody, causing the cells to adhere and to agglutinate. This ingenious method has been worked out in the case of the *incomplete* Rh antibody by Coombs, Mourant, and Race[14] in England, and has become widely known as the Coombs test.

(4) The fourth method used to demonstrate the presence of an *incomplete* Rh antibody deals with a change in the cell rather than in the antiserum. The Rh positive cell is exposed to the action of proteolytic enzymes, such as trypsin or papain.[15,16] Contact of only a few minutes is sufficient to change the surface of the cell so that the *incomplete* antibody will then cause visible agglutination even in saline solution.

Let us look at the difference between a *complete* and an *incomplete* Rh antibody in the light of Ehrlich's concept of the existence of a variety of antibodies, as contrasted with the orthodox unitarian concept of antigen-antibody reactions. In our specific example, we are dealing with one and the same single antigen, the Rh antigen, referred to as *D* in the English nomenclature, and as Rh_o in Wiener's nomenclature. We are concerned here with one antigen, and yet we are confronted with the occurrence of antibodies against this antigen which show totally different characteristics, depending upon the environmental circumstances. The differences then show themselves in the ease with which agglutination takes place, this being largely a function of the nature of the diluent. It was first thought that undiluted human serum would always do the trick, but in quite a few instances, agglutination could be obtained only as the result of fortifying the diluents with concentrated albumin solutions. Even with the introduction of albumin as a diluent, certain Rh antibody functions still remained undetectable except by the Coombs test. The different varieties of Rh antibodies can actually be separated by physical means, such as dialysis, provided a suitable Rh antiserum is used and the correct methods applied.[17] We can prepare protein fractions of Rh antisera in which the various kinds of Rh antibodies are present, if not completely isolated from each other, at least in different proportions. No wonder, therefore, that the existence of several orders of Rh antibodies has been suggested,[18] and it seems to me that there is every experimental reason to justify such a concept. It is sometimes rather difficult to decide where one variety of antibody stops and the second variety starts. We therefore prefer to visualize the existence of an antibody spectrum,[17] which is composed of a considerable variety of clearly different kinds of individual Rh antibodies, all exhibiting the same specificity directed against one and the same antigen receptor. There is experimental evidence which leads us to believe in the general occurrence of such antibody heterogeneity. It would seem justifiable today, therefore, to modify considerably the unitarian concept of antigen-antibody reactions, and to incorporate into the theory the idea of the antibody spectrum, as follows:

(1) The relationship between an antigen and its antibody is a specific one and can be explained only on a stereochemical basis.

(2) The combination between an antigen and its homologous antibody can result in precipitation, agglutination, lysis, phagocytosis, or anaphylaxis, depending upon the environmental conditions.

(3) There exists a spectrum of antibodies which might cover a wide range, as is the case in Rh antibody functions. This range might extend from the one extreme where the mere combination of antibodies with the antigens results in the formation of visible aggregation, to the other extreme where no change in diluents will bring about visible agglutination. The union of the antigen with the antibody can be demonstrated only by the addition of a second antibody which is directed against the globulin molecules comprising the first antibody.

In studying the occurrence of the variety of antibodies endowed with the same specificity, yet exhibiting different modes of action following union with the antigen, we observe what is sometimes referred to as the heterogeneity of the antibody combining site. In this regard, the question of the mono- or polyvalence of antigens and antibodies assumes major importance. It is my understanding that many immunochemists believe in the bivalence of the majority of antibodies. In observing antibody functions resulting in the mere blocking of the antigenic site without producing a visible effect, one could be inclined to attribute such a phenomenon to a univalent antibody. The easy transformation of *blocked* cells into *agglutinated* cells by simply changing the diluent, however, would shed doubt on the univalence of such an antibody. Ehrlich had already postulated the existence of both types of antibodies because what he called an *amboceptor* was actually a bivalent antibody acting as a bridge between two other substances. However, the concept of bivalence of antibodies, as we understand it today, was actually introduced by Marrack,[19] for Ehrlich considered only the antibody involved in lysis to be bivalent. The chemical analysis of precipitates resulting from antigen-antibody combinations has contributed a great deal to our present improved understanding of antigen-antibody unions. Heidelberger's work in determining the antibody nitrogen by the method of Kjeldahl has presented great potentialities for studying the quantitative relationship of antigens and antibodies. It is obvious from these studies that many antigens are *multivalent*, a concept which has helped to clarify many observations which could not have been interpreted at the time Ehrlich postulated his theory in 1897. This presentation does not intend to discuss the two major concepts of antigen-antibody combination which are still under consideration today. According to Bordet, a first stage consists of the coating of antigens with their corresponding antibody, followed by a second stage, nonspecific in nature, resulting in visible agglutination and precipitation. The lattice or framework theory of Marrack, Heidelberger, and Pauling, on the other hand, recognizes only one continuing reaction, the visible stage of the reaction being attributable to the continued agglomeration of smaller complexes into bigger ones, based upon the multivalence of antigens and antibodies.

Looking over the last 50-odd years which have elapsed since Ehrlich first postulated the side-chain theory, it is interesting and gratifying to note that many outstanding chemists have become interested in the problem. Ehrlich, though intensely interested and gifted as regards chemical concepts, was actually a physician and biologist by training. The field of immunology has developed in two somewhat different directions, the one direction represented by immunologists interested in biological and medical problems, and the other by a group concerned with the chemical analysis of serological problems. The second group, believing sometimes to stand on safer scientific grounds, is inclined to underestimate the efforts of the biological group whose main interest lies in the discovery of new biological phenomena. I should like to remind you of the fact that, since the time of Ehrlich, Behring, and Bordet, and mainly through their efforts, developments of immense significance have been made. An enormous number of blood transfusions, for example, are now given with ever-increasing safety without the help of a single Kjeldahl apparatus.

The surface of the red blood cell, we know today, is not as smooth as originally believed. In the discussion of the *incomplete* Rh antibody attention was called to another method which would allow some *incomplete* antibodies to act as *complete* antibodies, namely by mixing red blood cells with certain enzymes. Apparently, these enzymes destroy various points on the cell, possibly by a sort of leveling-off action, exposing the receptors normally hidden from the action of the *incomplete* antibody. Such a finding might suggest that the *complete* and *incomplete* antibodies are of different molecular shape, the *complete* antibody being longer than the *incomplete* one. Furthermore, the unevenness of the surface and the existence of *prongs* on the surface of the red blood cell would explain many mysterious phenomena which have thus far remained inexplicable. Coombs[20] in England is developing this concept by building chains between antigens and antibodies which, when long enough, finally result in agglutination of the cells involved. His success indicates the existence of hills and valleys on the surface of the red blood cell. What a surprise such a finding would be to Ehrlich and his co-workers, who had to explain the drawings of receptors as *symbols* of chemical groupings rather than morphological structures.

Antigenic Properties of Lipids and Polysaccharides

Ehrlich considered the specific relationship between antigen and antibody as limited to the antigenic grouping and its corresponding antibody. However, Forssman's discovery[21] of an antigen common to the blood cells of the sheep and the tissues of many *un*related animals, such as the guinea pig, the dog, the cat, the chicken, and certain fish, seemed to contradict Ehrlich's receptor theory for some time. Closer analysis of the problem revealed the amazing existence of an antigen occurring in unrelated animals, allowing the classification of the animal kingdom into two groups, one group (the guinea-pig type), exhibiting the Forssman antigen in one or several organs; the other group (the rabbit type), not containing the antigen. Interestingly enough, most of the animals of the latter group have an antibody in their serum directed against the Forssman antigen in sheep cells. In this respect they differ from the guinea-pig type of animals, whose serum does not contain this antibody. Again, we are impressed with the operating of the *horror autotoxicus* principle, inasmuch as animals either have the antigen in their tissues or the antibody in their plasma, but never both simultaneously.

Only proteins were considered to be antigens up to that time. Immunologists therefore experienced a second surprise when Forssman antisera proved to precipitate not only aqueous extracts of Forssman-containing tissues, but even the alcoholic protein-free extracts of those organs. Apparently the Forssman antigen belongs to the loosely defined group of lipids, though that statement might be somewhat debatable today.[22] Alcoholic tissue extracts containing Forssman antigen suspended in saline solution, however, failed to *elicit* antibody formation when injected into rabbits. These extracts constituted the first example of an antigen which could combine and precipitate with antibodies in the test tube without stimulating antibody formation. By a simple mixture of the Forssman lipid with a foreign protein, such as hog serum, Landsteiner was able to elicit antibodies against both constituents of the mixture by intravenous injection into rabbits. Substances of the Forssman type were called *haptens* by Landsteiner. Ehrlich and Morgenroth already felt that what they called an *ictus immunisatorius*, *i.e.*, an antigenic stimulus, was necessary for the elicitation of antibodies. Apparently lipids, by themselves, are lacking in this ability, but regain it when combined with a foreign protein, as in the admixture of hog serum. A hapten, then, may be called a substance which reacts specifically in the test tube with an antibody but is inadequate by itself for antibody production.

There is another example of a biologically important tissue-specific lipid, and that is the brain-specific lipid. The intravenous injection of rabbits with brain suspensions of different species will lead to the formation of antibodies which precipitate alcoholic extracts of the brains of all animals and birds.[23] The injection of fresh rabbit brain into a rabbit, however, fails to elicit the production of brain-specific antibodies, obviously because of the absence of the foreign protein which is necessary for the formation of the antibody. This statement holds true notwithstanding certain experiments according to which rabbit brain, after being autolysed for a period of time, seemed to gain some antigenic properties.[24] The introduction of Freund's method[25] of immunization by means of adjuvants created experimental conditions which apparently furnish the *ictus immunisatorius* necessary to elicit brain antibodies, even when brain suspensions of the same species are used. The resultant clinical manifestations referred to as *allergic encephalomyelitis* provide another most interesting tool for experimental study.

Complex carbohydrates assume an intermediate position between proteins which can readily act as antigens, and lipids which can act only as haptens. Heidelberger and Avery[26] isolated polysaccharides from encapsulated pneumococci which proved to be the bearers of type specificity. These carbohydrates give precipitation in the test tube when mixed with their corresponding antisera, but fail to elicit antibody production when injected into rabbits. Other animals, however, when treated with the same substances, will develop type specific immunity against infection with pneumococci.

The blood-group-specific antigens, A and B, also are carbohydrate haptens. When the term *blood groups* was coined, it was meant to characterize properties of blood cells only. Actually the entire human body is group-specific stigmatized,[27] and group-specific substances are constantly secreted in large amounts into the saliva and into the gastric juices of most individuals of the respective blood group.[28] Protein-free, water-soluble polysaccharides exhibiting the blood group-specific characteristics can be isolated from the body fluids of individuals of Group A[29] and Group B.[30] These substances when injected into rabbits fail to elicit antibody production, their behavior in this respect proving similar to that of the pneumococcal carbohydrates. However, when injected into human beings, they increase specifically their corresponding isoagglutinin, and therefore exert definite antigenic functions.[31] Again, the principle of *horror autotoxicus* manifests itself, since, for instance, an individual of Group A injected with A substance forms no anti-A antibodies. Only individuals of Group O and Group B when treated with A substance will respond with antibody formation, a procedure which is used for practical purposes for the production of potent test serum.

The definition of a hapten, therefore, is complicated, since the response depends not only on the nature of the material but also on the species of animal into which the material is injected.

Theories of Antibody Formation

Most of our discussion so far has dealt with that part of the side-chain theory which was concerned with the stereochemical nature of antigen-antibody combinations. This concept has withstood the test of time remarkably well. The second part, pertaining to the mechanism of antibody formation as a result of the secretion of receptors of pre-existing design into the circulation has also been attacked and criticized ever since the theory was expounded.

One of the objections to Ehrlich's concept is the observation that antibody formation occurs against a group of chemoantigens which are not present in living tissues. On the other hand, Buchner's original idea of antigens built into the antibody molecules has been definitely disproven. Important new ideas were contributed around 1930 by Breinl and Haurowitz,[32] by Alexander,[33] and by Mudd.[34] These ideas were later revised and extended by Pauling.[35] Pauling visualizes the formation of antibody molecules as occurring in close contact with the antigenic substance. The postulated mechanism is based upon a physico-chemical picture, and is described in terms of the coiling of a polypeptide globulin chain as influenced by its close proximity to the antigen. A complementary impression is thus produced on the gamma globulin molecule, which then detaches itself from the antigen, allowing repetition of the process. Since this theory does not adequately account for the persistence of antibody production in the apparent absence of antigen, and for the phenomenon of the anamnestic reaction, Burnet, using a more biological approach, has introduced some new and stimulating concepts.[36] The essential features of his theory remind us, in a way, of Ehrlich's concepts. Here, the idea of *receptor* is replaced by *adaptive enzyme*, and the idea of *horror autotoxicus* is amplified by the concept of the *self marker*. According to Burnet, the various cells of an organism must carry a means of identification which will protect them against the phagocytic activity of the organism, and also prevent the formation of antibodies against them. These distinguishing parts of the cells, and presumably also of dissolved antigenic proteins, are called *self markers*. An example of such a self marker would be the polysaccharide responsible for the ABO blood grouping differentiations. Another marker on the red cell would be some portion of the protein mosaic which would identify the cell as human rather than of another species. Burnet feels that a small number, perhaps five or ten, of such markers would suffice for each cell. The other part of the theory assumes the existence in the antibody-forming cells of normal enzymes whose configuration allows the markers of that same species to be recognized, thus preventing phagocytosis and antibody production. This recognition would stem from a complementary structure between the marker and the enzyme. When foreign material is introduced, adaptation of the enzymes to these markers takes place. These adapted enzymes have the property of self-replication and can continue to duplicate themselves in countless generations of daughter cells, even though the original antigen has completely disappeared. These replicas carry the specific complementary pattern, but not the original enzymic activity, and are liberated into the circulating fluids to become the antibody molecules. Burnet does not attempt to explain the mechanism whereby the adaptation takes place. As proposed by Campbell,[37] these two theories may be considered complementary if one accepts Pauling's suggestion on the mechanism of antibody formation, and if one considers such an antibody to be the primary adaptive enzyme of Burnet, which would then duplicate itself to produce circulating antibodies.

Obviously, the phenomenon of antibody production is still quite a mystery, as Bordet stated so many years ago. One of the great difficulties in studying the problem was ignorance about the site of antibody formation. Astrid Fagraeus' discovery[38] of the importance of the plasma cell as a site of antibody formation opens a promising avenue for the further experimental study of antibody formation. Nevertheless, the formulation of the side-chain theory, as stated by Ehrlich in 1897, represented an ingenious concept describing the stereochemical relationship between antigens and antibodies, a concept which stimulated, and is stimulating at the present time, a tremendous amount of research. What better comment can be made about a theory than that, for such a long period of time, it has challenged the minds and imaginations of investigators? For this reason, speaking for the spiritual children and grandchildren of Paul Ehrlich, we bow our heads in deep reverence at the celebration of the 100th birthday of such a master.

References

1. Ehrlich, P. 1897. Klin. Jahr. **6:** 299.
2. Bordet, J. 1899. Ann. inst. Pasteur. **13:** 225, 273.
3. Sachs, H. 1902. Beitr. Chem. Phys. **2:** 125.
4. Fischer, E. 1898. Z. physiol. Chem. **26:** 60.
5. Ehrlich, P. & J. Morgenroth. 1900. Berlin. klin. Wochschr. **31.**
6. Ehrlich, P. & J. Morgenroth. 1900. Berlin. klin. Wochschr. **21.**
7. Landsteiner, K. 1901. Wien. klin. Wochschr. **14:** 1132.
8. Ehrlich, P. & J. Morgenroth. 1901. Berlin. klin. Wochschr. **10.**
9. Levine, P. & R. Stetson. 1939. J. Am. Med. Assoc. **113:** 126.
10. Diamond, L. K. & N. M. Abelson. 1945. J. Lab. Clin. Med. **30:** 204; *Ibid.* **31:** 668.
11. Wiener, A. S. 1945. J. Lab. Clin. Med. **30:** 662.
12. Race, R. R. 1944. Nature. **153:** 771.
13. Diamond, L. K. & R. L. Denton. 1945. J. Lab. Clin. Med. **31:** 821.
14. Coombs, R. R. A., A. E. Mourant & R. R. Race. 1945. Lancet. **2:** 15; Coombs *et al.* 1945. Brit. J. Exptl. Path. **26:** 255; Coombs *et al.* 1946. Lancet. **1:** 264.
15. Pickles, M. M. 1946. Nature. **158:** 880.
16. Morton, J. A. & M. M. Pickles. 1947. Nature. **159:** 779.
17. Witebsky, E. & J. F. Mohn. 1948. J. Lab. Clin. Med. **33:** 1353, 1361, 1369.
18. Hill, J. M., S. Haberman & R. Guy. 1949. Am. J. Clin. Path. **19:** 134.
19. Marrack, J. R. 1934. The chemistry of antigens and antibodies. Med. Research Council (Brit). Special Rpt. Ser. **194.**
20. Coombs, R. R. A., A. N. Howard & L. S. Mynors. 1953. Brit. J. Exptl. Path. **34:** 525; M. H. Gleeson-White, D. H. Heard, L. S. Mynors & R. R. A. Coombs. 1950. *ibid.* **31:** 321
21. Forssman, J. 1911. Biochem. Z. **37:** 78.
22. Tomcsik, J. & R. Fischer. 1946. Schweiz. Z. Path. u. Bakt. **9:** 573.
23. Witebsky, E. & J. Steinfeld. 1928. Z. Immunitätsforsch. **58:** 271; E. Witebsky. 1929. Naturwissenschaften. **17:** 58.
24. Schwentker, F. F. & T. M. Rivers. 1934. J. Exptl. Med. **60:** 559.
25. Freund, J. 1951. Am. J. Clin. Path. **21:** 645.
26. Heidelberger, M. & O. T. Avery. 1924. J. Exptl. Med. **40:** 301.
27. Witebsky, E. 1927. Z. Immunitätsforsch. **49:** 517; E. Witebsky & K. Okabe. 1927 *ibid.* **52:** 359.
28. Schiff, F. & H. Sasaki. 1932. Klin. Wochschr. **34:** 1426.
29. Landsteiner, K. 1932. Science. **76:** 351; 1936. J. Exptl. Med. **63:** 185; K. Landsteiner & M. W. Chase. 1936. J. Exptl. Med. **63:** 813; W. F. Goebel. 1938. J. Exptl. Med. **68:** 221.
30. Witebsky, E. & N. C. Klendshoj. 1940. J. Exptl. Med. **72:** 663.
31. Witebsky, E., N. C. Klendshoj & C. McNeil. 1944. Proc. Soc. Exptl. Biol. Med. 55: 167.
32. Breinl, F. & F. Haurowitz. 1930. Z. physiol. Chem. **192:** 45.
33. Alexander, J. 1931. Protoplasma. **14:** 296.
34. Mudd, S. 1932. J. Immunol. **23:** 423.
35. Pauling, L. 1940. J. Am. Chem. Soc. **62:** 2643.
36. Burnet, F. M. & F. Fenner. 1949. The Production of Antibodies. MacMillan. London.
37. Campbell, D. H. & F. Lanni. 1951. The chemistry of antibodies. In Amino Acids and Proteins. Thomas. Springfield, Ill.
38. Fagraeus, A. 1948. J. Immunol. **58:** 1; A. Fagraeus. 1948. Antibody Production in Relation to the Development of Plasma Cells. Stockholm, Sweden.

EHRLICH AND PROBLEMS OF IMMUNITY

By Bela Schick
Mount Sinai Hospital, New York, N. Y.

On the fourteenth day of March 1854, a genius was born whose name was Paul Ehrlich. One hundred years have come and gone, but our medical thinking is still profoundly influenced by his life work. Ehrlich was a hard worker, indefatigable, supplying an enormous driving force in the institution in which he worked. He expected full cooperation from all members of his staff, inspiring them with his enthusiasm and with an inexhaustible fund of new ideas. From his early student days, his basic ideas in the field of research were so far-reaching that they defied complete exploration during his lifetime, many of them having to await amplification by later generations.

The fact that aniline dyes were capable of staining different elements of the cell impressed Ehrlich, for he realized the great significance of this phenomenon. It meant that there was an affinity between a chemical substance and different substances composing the cell, dead or living. This possibility gave him the idea of using dyes and other chemical substances to attach an invading germ responsible for disease, and to do so without damaging the tissue. The treatment of an infectious disease at the time of Ehrlich's studies was based on the use of an antitoxin in neutralizing the exotoxin produced by the invading germ.

He himself studied the action of ricin and abrin plant-toxalbumine, and the problem of producing neutralizing antitoxic substances. Behring had great difficulty in obtaining a high potency of the diphtheria-antitoxin, and it was Ehrlich who pointed out to him the solution of this problem. Only with Ehrlich's method was it possible to make the serotherapy against diphtheria effective: repeated injections of toxin, in increasing amounts, led to a higher concentration of antitoxin units. Ehrlich realized also that there must be an exact measurement of the potency of the toxin and of the antitoxin value before the treatment could be effective. The establishment of these measurements was essential for the study of many problems of immunity and for the treatment of exotoxic diseases. These studies led Ehrlich to the development of the famous side-chain theory, which for many years constituted practically the exclusive theory of immunity. I remember very well that, around the beginning of this century, when I became associated with the Serotherapeutic Institute in Vienna, all those who were working on problems of immunity were studying the book of Ludwig Aschoff, dealing with the side-chain theory of Ehrlich. Heated arguments *pro* and *contra* prevailed for many years before the controversy subsided. Even if portions of the theory are open to criticism and subject to doubt, the one thing that is certain is that the side-chain theory made many studies in immunity possible and was responsible for important discoveries. We know that, in conducting research, theories are necessary and useful. We also realize that theories may be wrong, and we must always be ready to modify and even to drop a theory if facts are found which contradict it. Ehrlich performed hundreds of experiments, some failures, others successes, in his effort to prove or disprove his ideas.

Interesting progress may result from experiments started on the basis of a wrong theory. Ehrlich fought for his theory, which was important to him, and in this way he stimulated new discoveries. As Michaelis pointed out "Discoveries never can perish or disappear, but will outlast all time." Clearly, it is of no importance that some people disagreed with the side-chain theory.

There is no question that facts are exceedingly important in research, but it is likewise of importance to have a leading idea behind any experiments that are made. I shall never forget a scene in *The Magic Bullet*, a motion picture portraying the life of Ehrlich. A representative of the German government had been sent to investigate the Ehrlich Institute in order to determine whether an application for a government subsidy should be granted. The investigator inspected all parts of the institute, observed the animal experiments and, as he was about to depart, assembled the entire staff. Then, standing in front of the staff, he asked: "What does Ehrlich do?" There was an embarrassing silence for a few seconds. Then a Japanese scientist, named Hata, if I am not mistaken, answered "Ehrlich thinks."

It is not my task nor my intention to enumerate all the magnificent accomplishments of Ehrlich. He is the father of hematology and of chemotherapy. He is the author of the famous adage "*Corpora non agent nisi fixata.*" The synthesis of salvarsan was the culmination of his remarkable career. Very fascinating were his studies with methylene blue, a real forerunner of the antibiotics of today. The term chemotherapy differentiates this form of therapy from the serumtherapy which, at that time, dominated the field of therapy.

I have already mentioned that Ehrlich studied ricin and abrin and their antitoxin. He realized that diseases such as diphtheria and tetanus damage the organism by an exotoxin, and that they were being correctly treated with a specific antitoxin. Nature accomplishes the cure in the same way. To imitate nature is logical. But diseases such as sleeping sickness, relapsing fever, malaria, or syphilis could not be cured by specific antitoxic sera. Another approach was necessary. Ehrlich conceived the idea of using a chemical substance which could attack specifically the invading microorganism without endangering the tissue of the organism. In this way Ehrlich formulated the concept of "*Therapia magna sterilisans.*" Many difficulties had to be overcome, among them the opposition and even animosity of expert chemists. But Ehrlich persisted in the pursuit of his ideas. Of the chemical substances constituting arsen compounds, he first used atoxyl, but it was found to be too toxic. He succeeded in changing atoxyl to an effective substance. In spite of the opposition of leading chemists, he was able to reduce atoxyl. Béchamp, a renowned chemist of that time, disagreed, but Ehrlich demonstrated that he could produce simple derivatives which became the basis of hundreds of new atoxyl compounds, and finally of salvarsan.

Further development of Ehrlich's ideas showed how correct he had been. Whereas not too many new effective antitoxic sera have been developed, chemotherapy has made great progress. The discovery, in recent years, of the therapeutic effect of the sulfonamides and of the antibiotics corroborates Ehrlich's idea of hitting the invading germ without too much damage to the tissue involved. Many of Ehrlich's concepts have had to be amplified in order to include new ideas, as, for instance, the bacteriostatic effect of sulfadrug. The specific effect of antibiotics upon different types of bacteria clearly demonstrates the importance of the chemical structure of the antibiotic used. Here, I wish to point out that we owe to Ehrlich the discovery that exotoxin can be made much less toxic by changing it to a toxoid. Ehrlich also established the fact that toxoids do not lose their antigen character, and therefore can be used for immunization. Further, we know that toxoids have made immunization not only safer but more successful.

Ehrlich also devoted himself to the problem of cancer, but his investigations in this field were unfortunately terminated by his early death. He did establish many interesting facts, among them the stimulating effect of the removal of the malignant tumor upon its remnants.

It is not generally known that much credit is due Ehrlich for the rapid acceptance of the discovery of the tubercle bacillus by Koch. Twenty-four hours after Koch published his discovery, Ehrlich, a master at staining blood cells and tissue cells, had applied his knowledge to staining the tubercle bacillus. Even today, Ehrlich's technique remains one of the best methods of visualizing the tubercle bacilli.

Science never stands still. Our search for knowledge must advance. But all who devote themselves to scientific research realize that they rarely have entirely new concepts or that they achieve results completely unrelated to past discoveries. Even if we change our ideas and have to tear them down, we shall still find useful bricks in the process with which to build up new structures of thought. We now know more about the existence of the different types of microorganism which secrete type-specific toxins. We know about specific carbohydrate groups in the composition of different antigens and antibodies. We have had much to learn about virus diseases and their pathogenesis, treatment, epidemiology, and prevention. A new world for research work was opened up with the construction of the electron microscope. Thousands of new problems are piling up before us; innumerable questions are waiting study. Chemistry and physics are now in the foreground of research methods.

It is astonishing to realize how little laboratory equipment Ehrlich used. Bottles of dyes and chemicals, test tubes and a Bunsen burner, bottles containing reagents and substances for the preparation of chemical solutions standing on some shelves along the walls, and cages for the animals: these items sufficed for his many experiments involving so many new ideas.

We have already mentioned the fact that Paul Ehrlich evolved a theory to account for antibody formation, his memorable side-chain theory. He was of the opinion that traces of each type of antibody are pre-existent in the organism as so-called receptors; that the receptor regenerates at an increased rate when the corresponding antigen is injected and becomes attached to the receptor. This part of the theory can no longer be accepted, since Landsteiner has shown that artificially created chemical substances induce the production of specific antibodies. But that part of the theory which defines the action of complement still prevails. The soluble antibodies of immune-serum are found in the globulin fraction. A hypothesis was therefore evolved that the globulins are the real antibodies. Their antibody function is owing to the adaptation of their shape to the shape of the determining group of the antigen; but the insoluble proteins of the connective tissue and of the smooth muscles also react with the antigen. It is difficult to judge these fixed antibodies quantitatively. Purified antibodies have all the characteristics and properties of normal globulin. A newer theory assumes that either the changes in the sequence of amino acids in the peptide chain or a different mode of folding of the peptide chain, brings about the antibody function of the globulin. But most likely other forces take part in the change. It is impossible to manufacture antibodies *in vitro* by slow denaturation of normal globulin in the presence of an antigen.

An interesting fact is that the antibody formation started by an antigen can continue after all antigen has disappeared. We find in clinical observation that the positive tuberculin reaction persists for many years after the original infection has been arrested. We assume that a small tuberculous focus persists containing living tubercle bacilli, so that some stimulation by an antigen is to be expected. It has been proved that an animal loses its positive tuberculin reaction several months after the last tubercle bacillus has been removed. The positive tuberculin reaction turns negative within one to two years after B.C.G. immunization. The infection is caused by an attenuated bacillus and it can be expected that in this infection the bacillus can be more quickly eliminated. The long persistence of immunity after smallpox and measles can be explained with the assumption that the virus survives within the organism. That the immunity following cowpox vaccination disappears within 12 years may be caused by more rapid destruction of the attenuated cowpox virus. Bacterial polysaccharides acting as antigens are stored in the organism of mice for many months. It may be that, in breaking down antigens, specific particles persist within the organism, but cannot be detected. The antigen must play a role in the mechanism of antibody formation. Pirquet and I assumed that foreign protein is broken down by a kind of digesting enzymes, which work in a way similar to the enzymes within the gastrointestinal tract. Foreign protein is broken down to amino acids. When foreign protein is injected parenterally it is not known whether the bricks of amino acids are used and, if so, how they are used for the synthesis of protein. Particles of the broken down protein may still have specific qualities reappearing in the newly synthesized antibody.

Great difficulty exists in answering the question as to the site of antibody formation: almost every organ has been considered as the site. The reticulo-endothelial cells have been particularly mentioned in this connection by some investigators. Lymphoid tissue and lymphocytes have been considered by others. Intradermally injected, an antigen produces high concentration of antibodies in the lymphnodes nearest to the site of the injection, whereas, at this time, only traces of antibodies are found in the blood and in other organs of the body. The last word concerning the site of antibody formation has not yet been spoken.

After the first injection of an antigen the antibodies do not appear until after a certain latent period, the incubation time. The latent period in man varies somewhat from that in animals and varies with different antigens. In a serum sickness of the human organism an incubation time of eight to twelve days is found after the first injection of horse serum. After the injection of diphtheria toxoid, ten days to three weeks may elapse, even though the dose is very large. Apparently the size of the dose has only a slight influence upon the length of the incubation time. We observe a shortened incubation period after a second and repeated injection. After reinjection of horse serum, immediate and accelerated reactions are seen. The immediate reaction is owing to the presence of antibodies which can interact with the reinjected serum, whereas the accelerated reaction occurs when the antibodies have disappeared, the organism then being capable of reproducing the specific antibodies more quickly than the first time. This deduction is true for antibodies used against protein substances such as horse serum, and is also true for antitoxic antibodies specific against a certain exotoxin such as diphtheria or tetanus toxin. Pirquet called this altered reactivity allergy. I have demonstrated that even in clinical diphtheria the specific antitoxin appears much more quickly upon a repeated infection. This observation of the rapid reappearance of specific antibodies became of practical value when the so-called booster dose was introduced to improve or to reinstate the declining or lost immunity against diphtheria and tetanus. As a rule the subcutaneous injection of the antigen is the best method to stimulate the production of antibodies. The statement that young animals are poor antibody producers has been published, but this assertion has lately been questioned. The influence of cortisone and ACTH upon antibody production is being studied, also the effect of these substances upon the interaction between antigen and antibodies, and upon resistance and immunity.

Of interest is the long persistence of type-specific pneumococcal anticarbohydrate within the organism, persisting much longer than diphtheria antitoxin. Reinjection or a booster dose of the pneumococcal type-specific anticarbohydrates has not the same beneficial effect as reinjection of diphtheria toxin or toxoid. Antibodies reappear, but the height attained is rarely equal to the original maximum.

That the type specific pneumococcal polysaccharides persist for such a long time within the organism was explained by Heidelberger by the fact that these substances are extremely resistant to an attack by enzymes, whereas toxins and toxoids, as typical proteins, are easily broken down. Nothing has yet been found in the make up of amino acids to set them apart from other proteins and to account for their highly dangerous properties. Like other proteins, diphtheria toxin is easily destroyed by proteolytic enzymes.

In our study of serum sickness, Pirquet and I showed that the foreign serum as an antigen is, to a great extent, retained in circulation during the incubation time, until antibodies and clinical symptoms appear. The antibodies remove the antigen rapidly. This observation was corroborated with the use of radioactive labeled substances (J 131). Our observations in serum sickness have been the basis for allergy. The symptoms of serum sickness were explained as the effect of the interaction of antigen and antibodies. Several clinical syndromes can be explained as allergic in nature, as for example the etiology of acute nephritis. Acute hemorrhagic nephritis is seen frequently in the third week after the onset of scarlet fever. I pointed out that the first phase of scarlet fever is the effect of the primary toxin derived from the specific streptococcus or from a virus. This primarily toxic substance sensitizes the organism, and particularly the lymph nodes and the kidneys, to itself. Antibodies need between two to four weeks to develop. They appear in the circulation and interact with the still-present or produced primary toxin. This interaction is responsible for the clinical symptoms showing the flare-up in the lymph nodes and in the kidney, which we called postscarlatinous lymphadenitis and nephritis. Many features of the reaction resemble serum sickness.

A similar explanation is applied to the peculiar behavior of rheumatic infection. The disease is preceded by a single or repeated attacks of tonsilitis or upper respiratory infections. These preceding infections are a sensitizing factor for the onset of rheumatic symptoms. They appear after an interval of 14 days. The rheumatic symptoms are allergic in nature. Other clinical pictures have been declared allergic. Rich and Gregory demonstrated, in several human diseases, histologically allergic features resembling allergic reactions in rabbits after an injection of foreign protein. The so-called collagen diseases, lupus erythematous, periarteritis nodosa, *etc.* are classified as belonging to this group. This classification gave the basis for their treatment with ACTH and cortisone.

In explaining clinical symptoms as allergic, it should not be forgotten that every infectious disease must show an allergic feature because each infection presents an antigen producing specific antibodies which interact with the antigen. The symptoms of the disease are the consequence of this interaction. In diseases caused by endotoxic substances, these substances are set free by the destruction of the bacillus by bacteriolytic antibodies. We pay with the disease for this defense mechanism. Immunity is established because the antibodies are retained. Even after these antibodies have disappeared, the organism has the potential ability to reproduce specific antibodies more quickly than the first time. The amount of liberated endotoxin will be smaller. As a consequence, the disease will be milder and may not be detectable even clinically. I proposed to call this form of beneficial allergy "physiological allergy."

Only an exotoxin produces specific neutralizing antibodies. No satisfactory antiendotoxin is known. The termination of an endotoxic disease can be accomplished only by killing the invading germ. We produce antibodies through a mild infection. If antibodies are present they stop a repeated infection. Thus immunity rests on allergy. Immunization by vaccination with attenuated pathogenic germs is an effective preventive measure leading to immunity. There is no immunity against an endotoxin. In this respect, an endotoxin resembles the foreign protein in serum sickness. The intermediary products arising from the interaction between antigen and antibodies are toxic. There is no immunity against this toxin.

In serum sickness, we see another symptom which has clinical importance. There is a decided increase in the intensity of the reactions in cases of reinjection of serum. This hypersensitiveness may not only reach an annoyingly high degree, but may even be a threat to life. The classical animal for studying this form of allergy, the guinea pig, shows this hypersensitiveness to an extreme degree. It dies after an intravenous reinjection in shock resembling asthma.

This hypersensitive reaction is called anaphylactic shock. Observation of these effects gave a clue to understanding the allergic basis of asthma and of many other clinical pictures attributable to protein hypersensitiveness. These effects are seen in food allergy, hay fever, pollen asthma, *etc.* These forms of allergy are not beneficial. They are a pathological form of allergy. No immunity exists against these substances. There exist two ways of treatment: the one is to avoid the intake of the offending foods and to avoid contact with the offending substances; the other way is to desensitize, through injections of the allergens, in slowly increasing doses.

Analysis of the chemical constitution of toxin and antitoxin has not yet been achieved. We know that antibodies are protein substances having all the criteria of normal protein. The specificity can be detected and proved only by biological methods. In the case of antibodies we have the difficulty of finding that they exist in serum, from which it is impossible to remove all nonspecific substances. In the purification of exotoxins the situation is more favorable because exotoxic substances are secretions which are obtained from bacteria growing on culture media. We are dealing with smaller amounts of substances not belonging to the toxin. More difficulty is encountered in the study of the purification of an endotoxin, which is set free only after destruction of the germ. Pappenheimer, Jr., studied the purification of diphtheria toxin, which he greatly improved. However, even after very far-reaching purification, some protein is still present.

Our knowledge of the chemical composition of toxins is, unfortunately, still limited. It is even more limited as far as toxins in virus diseases are concerned, because the mechanism of infection is still obscure. The virus circulates in the blood stream for short time only. It attacks the cell itself, settling either in the protoplasma or in the nucleus. The virus multiplies within the cell, interfering with its metabolism. As a result, the cell dies and the virus then has the opportunity of entering the circulation again. A therapeutic agent will have great difficulty in reaching the intracellularly located virus, and will be unable to attack the intranuclear settled virus. The time of spreading may be the time when therapeutic agents are most effective.

We know that antibiotics, so efficacious against different bacterial infections, are much less effective, or not at all effective, against diseases attributable to a virus. In connection with this discussion, it is interesting again to mention the fact, also discovered by Ehrlich, that antibodies are present in the globulin fraction of the serum and, as we now know, in the gamma globulin part. Burton, Janeway, I, and others found individuals who had no gamma globulin, or almost not any of this factor, in their sera. Some of these cases had a very low immunity against infectious diseases. These individuals suffered several attacks of pneumonia or other infections of the respiratory tract, and even several attacks of meningitis. An injection of gamma globulin every month in relatively small amounts improved the resistance of children to these diseases. There apparently exists a defect in the synthesis of gamma globulin. There are individuals who have fluctuating amounts of gamma globulin accompanying parallel changes in resistance.*

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*A case known to me enjoyed excellent health. This is not, however, the appropriate time to explain th exception.

The lack of effect of antibiotics upon virus diseases gave me the idea of using gamma globulin in infectious diseases where antibiotics have been tried without satisfactory results. With Harris as a collaborator, I was able to show that gamma globulin was of decided benefit in cases where antibiotics had failed. That gamma globulin has an antiviral effect is known. It is used against virus hepatitis and prophylactically against poliomyelitis. As the prophylactic effect is only temporary, it is clear that gamma globulin is not the last word in preventing poliomyelitis. Active immunization is necessary, just as in diphtheria, in tetanus, and in viral diseases such as smallpox.

I have now come to the final words of my discussion. One hundred years have elapsed since Ehrlich was born. He not only created the groundwork which led to our full understanding of toxin, toxoid, antitoxin, and immunity, but he also showed the way for further study, so that we can still continue our research along this road. Many byways have been opened since his time which lead to many new discoveries. Although Ehrlich concentrated at the height of his lifework on the chemotherapy of infectious diseases, we should always remember that he started with the study of antitoxic sera. Ehrlich realized the limitation of antitoxic serotherapy and developed his own original ideas of chemotherapy. If he were alive today, he could enjoy the satisfaction of seeing how correct his prophetic vision was. The genius of Ehrlich is still illuminating our research in immunity.

PAUL EHRLICH AND THE CANCER PROBLEM

By C. P. Rhoads
Sloan-Kettering Institute for Cancer Research, New York, N. Y.

We are concerned, in this centenary year, with the impact, potential and actual, of Ehrlich on the scientific endeavor of the present day in the field of cancer research. In order to present the case adequately, however, it is necessary to emphasize those basic principles of procedure, rather than individual discoveries, which were the product of his genius and to indicate how they pertain to modern medical studies.

It is conventional, in discussing any distinguished figure, to refer principally to his outstanding personal characteristics, especially if they are unusual. This procedure is followed, one must presume, in the belief that the reader will be inspired to emulate the described traits of character and so will succeed in achieving equally significant ends. It is, however, more important in stimulating advance to establish the intellectual processes, the pattern of thought, by which were accomplished the results which account for distinction.

Thanks to modern interest in scientific progress and to the ease of today's communication, the name of Ehrlich is now widely known. Most people associate with it the mental image of a lovable and eccentric scientist, obscurely and absent-mindedly, in a murk of cigar smoke, laboring almost single-handed to produce the "magic bullet" of salvarsan, capable of striking at the heart of the most dread disease of the time.

Equally accepted is the concept of the empiric approach embodied in the cabalistic figure "606." Any student can conjure up the scene of a stooped, bearded, and bespectacled figure in the dingy laboratory of the Victorian era, methodically preparing and testing hundreds of compounds on a completely trial-and-error basis. This picture has some slight literal ground in fact, but is fundamentally erroneous, and indeed seriously misleading. The accomplishments of Paul Ehrlich, so lauded and so revered, and at times so reviled, were the consequence not alone of odd personal traits, blind persistence, attractive eccentricity, or solitary genius.

Furthermore, Ehrlich was an empiricist only when a rational approach required empiricism to solve its problems. His contributions were of basic intellectual principles, including those which made chemotherapy possible, together with the persistence, born of conviction, which made it inevitable. Many investigators, before and since Ehrlich's time, have made basic observations, and others have belatedly turned such observations to practical and useful ends. Few men have done both, and in Ehrlich's capacity to do so lay this man's great genius.

Finally, Ehrlich was anything but single-handed. If ever there was teamwork in research and ever firmly directed investigation, it was in his laboratory, and, moreover, in every laboratory of the time. The conviction that anything was possible ruled the day; scientists were proud servants of society, and they prided themselves on seeking to achieve desirable and practical ends.

Close examination of the facts indicates clearly that Ehrlich's accomplishments, which are the foundation of much of modern medicine, are the consequence in part of personal heritage, in part of individual brilliance, and to a great extent of the total scientific picture of the time, a picture composed of many elements and unique advances. This period was the golden age of German intellectual supremacy in the natural sciences, an age engendered by the revolution of 1848 and nurtured by a new sense of public service inherent in the trend toward socialism. It was a period in which the democratic process functioned within a monarchical framework and a tradition of rigid discipline inherited from Frederick the Great. This curious coalescence of the old and the new in thought and practice was leavened, in terms of scientific advance, by the almost incredible accomplishments of a great chemical industry. It was linked, furthermore, with an appreciation of the accomplishments made possible by proper organization of a scientific effort as established in the institute system. This discussion, then, will be focused on the reasons why Ehrlich succeeded in fields where so many had failed, with consideration of how modern society can profit by an example set a century ago.

Every biographer benignly indicates, with a quasi-paternal tolerance, the single-mindedness of Ehrlich, who began his scientific life with the application of a chemical technique: that of staining biological materials with aniline dyes, an occupation he continued almost until the day of his death. Such complete devotion to what would appear superficially to be a restricted and unprofitable pursuit, actually is the answer, if taken in the proper frame of reference, to the ability of this individual to accomplish so much. It should be recalled, however, that had it not been for the existence of a great chemical industry, one having a brilliant tradition and founded on the most basic organic chemistry, on the most rigid observance of technical procedure, and on the strictest disciplines of investigation, there would have been no dyes with which Ehrlich could work. Furthermore, had it not been for the public conviction that chemistry would yield cures, there would have been no institute such as the organization in which he worked, an institute devoted wholly to chemotherapy, one in which his endless patience and devotion could have full play. And, finally, had there not existed in the hospital system a controlling precedent which vested executive power for policy in the hands of the clinical investigators, the basic observation of Ehrlich could never have been brought to fruition in terms of the control of disease in human beings.

In summary, the setting into which Ehrlich was born was ideal for the man. Contacts, tradition, precedent, government, medicine, and industry all made their contribution—a combination which has rarely been achieved subsequently. Perhaps this fact is reflected in the productivity of present-day medical research. It would be well if we could draw from Ehrlich and his times a lesson in those principles of procedure which were the essence of their success and apply it to the acceleration of the progress of the present day.

What, then, was available when Ehrlich began his work on tumors? He, as a person, had acquired the unshakable conviction that organic chemicals could be found which would exert essentially any kind of selective effect on one or another type of cytologic unit, depending upon the ability of that unit preferentially to bind the chemical and so to permit the chemical to operate in a fashion different from the way it operates on any other cell type. This conviction stemmed from his original observations on staining and the later demonstration that methylene blue would selectively color nervous tissue *in vivo*, perhaps one of the most significant, basic, and revolutionary demonstrations of modern biological science. It was, in fact, the milestone which marked the end of Galenic philosophy and the triumph of the Paracelsan principle: the introduction of organic chemistry to biological study, and conversion of both to therapeutic success.

In retrospect, almost anyone can understand that if one dye will stain one tissue, and no other, in the living animal, inevitably other dyes can be found or made which will exert specific effects upon any group of specialized cells. This must be the case, whether they be external invaders, as bacteria, parasites, viruses, or internal deviants from the primordial oöcyte. Obvious as this may be as a triumph of hindsight, it was singularly unappreciated in Ehrlich's time. It remains to this day a fact rarely accorded its true importance. If any trait of Ehrlich were to be selected as his outstanding characteristic, in terms of its contribution to his scientific triumph, it would be this ability of his to see clearly the implications of a simple observation, and to see these implications so clearly as to provide a degree of grim determination, of unshakable resolution, to convert the principle to a major social advance. For the observant eye, it was only a minute step from selective dyeing to selective chemotherapy. To the defeatist, to the oppositionist, or to the reactionary, such a step is one he cannot take, and therefore one which he will never admit anyone else can take either. Not only is the mental transition wholly impossible to such a type, but, lacking it, he also lacks the fortitude required to convert principle to practice.

The first principle demonstrated by Ehrlich, then, a principle equally applicable today, is that of chemical specificity of cytologic structure, and the first requirement is the will to endure all the vicissitudes implicit in the conversion of this principle to therapeutic advance of any type desired. To generalize, one can say that the two major contributions of Ehrlich to contemporary science were the observant eye and the indomitable spirit. These characteristics are less the traits of the academician than they are those of a character in which the interests of his fellow men transcend those of personal glory. It is important, then, for a scientific community, if it would benefit by the lesson of the man whose centenary we celebrate, to recall that character contributes as greatly to scientific achievement as does erudition.

A third outstanding aspect of Ehrlich's genius was the ability to employ for his purpose essentially any type of scientific tool required. If the desired chemicals were not at hand they could be synthesized, if not by him, by those more skilled than he. Such resources were available to him through his manifold personal friendships and through the vigorous but disciplined national scientific industrial organization of the day.

We have established the unique characteristics of Ehrlich as vision and as the determination born of a sense of social responsibility. We have indicated that these characteristics were able to achieve revolutionary medical advance because of a public conviction that advance was possible; because of facilities which permitted single-minded concentration upon that advance; because of an industry which could and did assist liberally; and because of a medical organization steeped in the tradition of investigative medicine.

Ehrlich began his work on tumors, about 1900, in the conviction that the side-chain theory of specific chemical fixation to specific cell constituents might well apply to cancer control. Obviously, this concept was the application of his experiments on selective staining to the observations of Mueller and Virchow on neoplastic cells. The suggestion was unavoidable that cancer cells, as unique structures, might have constitutional characteristics which which would make them susceptible to specific chemical restraint or destruction. It is obvious that Ehrlich recognized the neoplastic cell as an etiologic entity in itself, one to be handled and studied as a bacterium and not one for which some other etiology might be sought.

We must presume that Ehrlich turned to cancer research as to a field unexplored up to that time, susceptible to major advance because of the availability of the transplantable mammary tumor in the mouse for exact, reproducible, and quantitative study. Clearly he viewed the cells of this experimental tumor as biological units subject to procedures as exact as those employed with the tubercle bacillus, the diphtheria bacillus, or ricin. Obviously, his experience either in staining or in immunology, or both, might be pertinent; neither experience had been applied to the cancer problem.

The whole concept of the transplantable tumor cell as an infecting micro-organism was cardinal to the study of cancer in Ehrlich's day and, some feel, is of equal importance today. This concept is particularly noteworthy because it developed in an era when the idea of disease as owing to micro-organisms invading the body from the external environment was transcendant. It is further noteworthy that Ehrlich's athreptic theory of immunity, although it turned out to be erroneous, was essential for the productivity of his studies of cancer. This concept was that invading micro-organisms, or their products, fasten on to anatomical structures tiny arms called "receptors" in the affected body cells. The foreign substances, if they exerted toxic effects, were presumed to do so by blocking the receptor whose normal function it was to carry into the cell needed nutritive elements. In modern terminology, the toxin was thought to act as an antimetabolite. Conversely, as immunity developed, it was presumed to be attributable to the presence in the blood stream of excess receptors produced by the cell in the function of its defense mechanism. These excess receptors, freely circulating, were supposed to render invading organisms innocuous by fixing on to their receptors, in turn, and so blocking off their ability to derive nutrition from the fluids of the body in which they would normally multiply. Clearly, the antimetabolite principle was conceived of as applying both to the action on the body cell by the invading micro-organism and also to the action on the invading micro-organism by the protective units produced in the body.

Since cancer cells were seen by Ehrlich as analogous to bacteria, as invading micro-organisms, it was natural for him to explore the possibility of inducing immunity to them. Under his theory, this possibility would be equivalent to starving them out, and we can assume that he thought that, if it could not be done by immune bodies, it could be done by chemicals acting in similar fashion with an equivalent specificity.

As far as can be ascertained, Ehrlich's interest in the cancer problem, though beginning with the concept of the cancer cell as a micro-organism, became really active when he saw the characteristics of these cells change with persistent transplantation. Preliminary observations satisfied him that the spontaneous mammary tumor of the mouse had a well-defined tendency to metastasize, even though it grew rather slowly. Persistent transplantation clearly gave a heightened growth capacity but also a decreased incidence of metastases. This could only suggest, to a mind as acute as Ehrlich's, the possibility of immunizing with tumor so modified by repeated transplantation as to be analogous to the attenuated bacterial culture which retains its antigenic capacity but is no longer infectious. This concept was, of course, the principle so clearly established by Pasteur. The possibility led to an intense interest in the transplantation of animal tumors and to a study of the changes consequent to this procedure when often repeated under varying conditions. One can well imagine the excitement, as the work continued, when the rapid increase in incidence of takes and rates of growth was seen. It was undoubtedly even more exciting when the unequivocal morphologic change was observed which took place in the cytologic composition of the transplants. To observe the shift from an obviously epithelial tumor to one which resembled in every respect a growth of mesenchymal origin, a sarcoma, was impressive indeed.

Not only did the transplanted tumors change in form and function, but suggestions appeared of an effect of the transplant on the tissues of the host. The evocation of blood supply and stroma was obvious enough, but to observe a change in the appearance of these structures apparently consequent to an action of the tumor on them was reminiscent of the action of bacterial toxins. Here was an analogy indeed, one which made more likely the possibility of inducing an active immunity. And then (shades of Ehrlich's first triumph, diphtheria therapy with antitoxin!), perhaps there could be developed some kind of cancer therapy with induced antibodies.

The early experiments on immunity were stimulating. It was quickly shown that in the case of some tumors a second transplant would not take if an original one were present and slowly growing, or had regressed. This finding led naturally to the employment of the best analogue of an attenuated bacterial culture, which was at hand in the transplanted tumor material. This material was a growth which had become hemorrhagic and so presumably had a high content of poorly viable or nonviable cancer cells which might still serve as good antigens. Rather conclusive studies showed that this was the case. The inoculation of the hemorrhagic tumors did not result in tumor growth, in many instances, but did so modify the recipient animal that the subsequent implantation of viable tumor fragments only rarely gave takes, though similar fragments grew regularly in animals not previously treated.

It should be recalled that these investigations took place in an era when men of great authority, such as Cohnheim, held strongly to the opinion that neoplasms were attributable to the growth of embryonal rests. The ability to immunize with weakly viable tumors led naturally, then, to the idea that perhaps normal embryonic tissue would function as an antigen evoking antitumor antibodies. This idea was tested with some success. Both embryo extracts and normal blood, when injected, appeared to render some normally susceptible animals partly or completely protected against subsequent implants of vigorous tumor. This fact has been long forgotten, although it crops up from time to time in the modern literature of cancer research. Studies have been reported only recently by McDowell and by others on immunity to transplantable leukemia induced by treatment with normal tissue or its extracts.

As the work went on, new facts developed which rendered the studies on immunity of doubtful practical application. Principally troublesome was the great variability of the experimental results, and particularly disappointing was the observation that, in many instances, second implantation would not only grow but would actually enhance the ability of the original transplant to multiply.

The studies on nutrition are well epitomized by the results in three series of animals, as follows:

(1) The first group was put on an almost starvation diet for several weeks, and the transplants would not take, or would take very slowly. The semi-starved animals, however, lived longer than the well-fed controls.

(2) In the second group, the limitation of food intake began only several days after the transplant. If the food intake was very small, the animals died very rapidly. This result was explained as proving that the tumor appropriated most of the available nutrients and thus deprived the organism of any response.

(3) In the third group, the limitation of food intake was also started several days after the transplant, but to a much less extent. As a result, there was an inhibition of tumor growth. These animals lived longer than the normally fed controls.

These results were interpreted as indicating three possibilities: (1) a transplanted tumor finds all the necessary nutrients and will grow; (2) a transplanted tumor will not find any nutrients which will fit its receptors, and will die; and (3) the tumor will find nutrients which will not fit its active receptors but may fit some of the latent receptors; if the active receptors disappear, the tumor may achieve the ability to grow.

Whatever the facts may be (and no better explanation is at hand in 1954) it is utterly amazing that, after 50 years, the specific chemical requirements of neoplastic cells, analogous to nutrition, so strongly emphasized by Ehrlich, so apparent from their special staining characteristics, and so long denied by the intransigent, should now be the foundation of modern cancer chemotherapy based upon the principle of antimetabolite action.

The apparently contradictory results obtained with the three series of animals led to an enormous amount of speculation on the part of Ehrlich and his co-workers in an effort to arrive at an explanation. Consistently invoked were the athreptic theories of the chief. If a second transplant failed to grow, it was argued that the first had exhausted the supply of some essential nutrient. If pretreatment accelerated the growth of the second transplant, it was held that the original tissue had supplied something needed for the growth of neoplastic cells. This promptly led to efforts to transplant tumors of one species into others and the inability to do so was explained again on the basis that essential nutritive constituents were unavailable. An important contribution to this subject was that of Murphy, who made the observation, recently rediscovered, that the treatment of an animal by X rays rendered it susceptible to the transplantation of neoplastic tissue of a different species, hence nutrition could not be involved. These studies led to a prodigious scientific altercation between two factions, the one school holding that the demonstrable immunity had nothing to do with nutrition, but rather resembled the reaction of antibodies to a bacterial toxin, and the Ehrlich group persistently holding to "athrepsia."

The effect of multiple implants of tumor tissue was further examined by excising one before the second was made. When this procedure was followed, with proper timing, the second implant would not grow. In the course of these studies, however, a remarkable observation was made. If the excision was incomplete and tumor cells were left at the site of the original implant, the second implant would grow. Two explanations of this result were offered. The one was that the recurrence soaked up the antibodies and so permitted the growth of the second transplant. The other, as might be expected, was that the persistent growth provided some nutritive material needed by the second inoculum. The conclusion drawn from these studies by Ehrlich, however, was that neoplastic cells have peculiar avidities for some ill-defined but essential component circulating in the host. The recent extensive studies of Mider and his colleagues, 50 years later, have provided unequivocal proof that this postulate was correct. In these studies, in which a tumor of high growth capacity is employed, the neoplasm increases rapidly while the host animal shrinks. Feeding of the host on a caloric content of tumor tissue equivalent to that of normal diet restores the lost weight. Clearly, the neoplasm has withdrawn from availability in metabolism some essential constituent which is today no better defined than it was in Ehrlich's time.

Persistent belief on the part of Ehrlich that neoplastic cells were different from normal cells and were the invaders to be destroyed if the disease were to be cured led to an interest in the differential sensitivities to physical agents of cancer as compared with normal cells. Ehrlich showed, fairly conclusively, that the neoplastic cell is injured by elevated temperatures, which are less inhibitory to normal cells, an observation which has had confirmation in two laboratories within the last two years. Braun has shown that the cells of certain plant tumors can be rendered more nearly normal by exposure to elevated temperatures, and Lettré has demonstrated the sensitivity of certain mammalian tumor cells to temperature change.

An important thesis concerning the neoplastic process was its division by Ehrlich into two phases: induction and growth. It is unclear how he came to this important conclusion, since he had no experimental means for tumor induction. It is interesting, however, that every study since his time has established the correctness of his postulate. Particularly revealing are the recent studies of Klein in plant tumors induced by *Phytomonas tumefaciens.* Here something not morphologically perceptible occurs in a well-defined time interval during which the bacterial culture is in contact with the plant cells. The tumor occurs long after the bacteria have been removed. Induction is clearly a separate process from cell multiplication.

Most interesting and least pursued was a minor observation which, if perceived at its true value, might have had important consequences. This was the observation that the transplants of the mammary tumor took only poorly or not at all in pregnant animals. It was easy for Ehrlich to explain this phenomenon on the basis of a demand by the embryo on the host for essential nutrients, a demand which starved the implant. He should have known, however, and apparently he did not know, of the studies of Beatson, published in England a number of years before, on the effect of ovarian removal on mammary cancer in women. The failure to pursue this suggestive lead was clearly owing to limited knowledge of experimental endrocrinology. The time was not ripe for the investigation indicated.

Ehrlich's final contribution to the study of cancer was his consideration of the cause of the neoplastic process. He persistently held to the thesis that these cells, so aggressive and so destructive, were the product of some constitutional cause. He envisioned clearly the possibility, undoubtedly because of his knowledge of acquired resistance by bacteria to injurious chemicals, that some process within the body which lowered the "vital energy" of certain cells would force them to acquire unusual properties, which in turn made them resistant to those forces which normally limit cellular growth and reproduction. He saw the neoplastic cell as an adaptive resistant form, its latent functions being brought into effective action by adverse environmental circumstances. It is impressive indeed that over a 50-year period of cancer research, no better theory has yet been found.

THE CHEMOTHERAPY OF TRYPANOSOME INFECTIONS

By C. H. Browning
University of Glasgow, Glasgow, Scotland

Paul Ehrlich and his school, in the decade 1904–1914, established that branch of pharmacology which Ehrlich called chemotherapy. It may be described as the treatment of infections by chemical agents which so damage the vitality of the parasites as to lead to their destruction. In recent terminology, Ehrlich's aim was to obtain drugs with selective toxicity for the pathogens. Isolated attempts at the drug-therapy of infected animals had been made before, but it was Ehrlich who developed "therapeutic biology" on experimental lines. He chose to start with trypanosome infections, and the body of knowledge thus established has proved widely applicable in all fields of chemotherapy thus far explored. The beginnings of his work are therefore worthwhile tracing in detail; they give an insight into the man. There were several good reasons why Ehrlich should have concentrated on chemotherapy at that time and why he selected trypanosomes for study. The serious limitations of immunotherapy had become apparent and it was obvious that many important infections could not be cured by such means alone. Among these infections was trypanosomiasis, which had recently wrought horrifying ravages in Africa. It had been shown that certain species of trypanosomes could be readily accommodated to mice and rats, in which they produced a rapidly fatal infection with regular septicemic course. Laveran and Mesnil had found also that arsenious acid, known to the explorer Livingstone as of some value in tsetse-fly disease, banished the parasites from the blood, although cure was seldom achieved. The animals died of the infection if treatment was stopped, or of arsenic poisoning if it was continued. One can readily imagine what a stimulus these facts were to Ehrlich—an infection of constant behavior in a convenient host attributable to a labile morphologically well-organized microparasite, with every indication that complete cure by a drug, though not yet achieved, was a distinct possibility. Also, there were the encouraging results obtained with Guttmann in a few cases of endemic malaria treated with methylene blue in 1891. It can be confidently stated that, but for Ehrlich, progress in this field would have been slow. Advance depended on the service of synthetic organic chemistry subordinated to biological aims. Probably no other biologist possessed Ehrlich's knowledge of the rapidly advancing domain of organic chemistry, especially in the dyestuff field, or could have applied the two disciplines to the single problem. Neither were there then, nor for long afterward, able chemists who counted it worthwhile to risk an academic reputation on what must have seemed a mere scientific gamble in an unknown field.

Ehrlich brought to this subject the knowledge and outlook of *Das Sauerstoff-Bedürfniss des Organismus* and his studies on immunity. In the former, he had tackled the problems of measuring the affinity of living tissues for oxygen by injecting dyes capable of reversible reduction to leuco-compounds and had shown astonishing skill in devising experiments and speculative daring in interpreting them. Ehrlich's studies on immunity had widened his conception of the capacity of the tissues for fixing substances biologically important, and had impressed him with the great specificity of such processes. In 1885, in *Das Sauerstoff-Bedürfniss*, side chains of protoplasm are mentioned and, in 1900, the term receptor is used to describe the combining group or side chain of the protoplasm molecule which anchors a substance such as a toxin, this mechanism being held identical with the mechanism subserving cell nutrition (Ehrlich and Morgenroth). Langley, the physiologist, later independently postulated "receptive substances" of the tissues for binding certain foreign compounds, which greatly gratified Ehrlich. Only recently, however, Whipple has proved that assimilation of protein molecules takes place by their direct passage into cells and that a similar exit occurs. This substantial evidence disposes finally of the gibe of fantasy directed at Ehrlich's receptors. In connection with chemotherapy, Ehrlich distinguished from the nutriceptors of the parasites, akin to those involved in antibody mechanisms, the chemoceptors which fix drugs. Also, he attributed to the drugs toxophore as well as haptophore groups, on the analogy of toxins.

The first product of chemotherapeutic investigation was the *Farbentherapeutische Versuche bei Trypanosomen Erkrankungen* by Ehrlich and Shiga (1904), which describes the original example of *therapia sterilisans magna*, because a single subcutaneous injection of the dye trypan red, one of the bis-azo (Congo red) series, sterilized an infection with *Trypanosoma equinum* in a mouse when administered 24 hours before the death of untreated infected control animals, *i.e.*, at a time when the blood was swarming with parasites. Ehrlich states that a large number of substances were tried without effect until a congener of trypan red showed some action. It was, however, too little soluble for practical use, and then his friend A. Weinberg, in collaboration with K. Ullmann, prepared trypan red. So far, apart from the natural "antibiotic" products, all the most effective chemotherapeutic agents have been obtained on these lines: first, the discovery has been made of a substance having some activity; and then the synthesis and comparative study of its related compounds has followed. The utility of every drug is limited by its toxicity for the treated animal, hence Ehrlich's first steps with each compound were: (a) to determine the minimum lethal dose for the host; (b) to find whether the maximum well-tolerated dose has the desired therapeutic action; and, if so, (c) to ascertain the minimum curative dose. The ratio of the first to the last of these quantities is the "therapeutic index," which enables substances to be compared. The introduction of various practically useful modifications into this scheme has not impugned its common sense: for example, the estimation it gives of the minimum lethal dose for 50 per cent of animals and of the minimum effective dose (which either "suppresses," *i.e.* prevents, infection or cures, or banishes trypanosomes temporarily from the blood) for 90 per cent; and the use of statistical controls of significance.

Early in his work, Ehrlich concluded that, as his deduction was later formulated, *corpora non agunt nisi fixata*. This generalization expresses for pharmacology a law which Grotthus and Draper had formulated for photochemical effects about 100 years before. The statement was not immediately welcomed, however, for in 1910, Ehrlich wrote in the preface to his book written in collaboration with Hata "for 25 years (*i.e.* since the time of *Das Sauerstoff-Bedürfniss*) I have striven hard to translate into practice the principle that a drug can act only on the bodily systems by which it is taken up, and now at length my sustained labor has reached a result for therapy. For long I stood alone with my views and efforts; only my cousin, Carl Weigert, too soon dead, again and again urged me on in face of discouraging failures." It has sometimes been overlooked, especially by those possessed of a mission to explain the wherefore of pharmacological phenomena, that the first and paramount necessity has been, as it still is, to secure really effective drugs. Ehrlich recognized this need clearly. He may consequently, at times, have been somewhat impatient of the pursuit of biological phenomena met with in the course of his work, which absorbed energy required for that aim. But the value of the observations made in this connection was quickly appreciated by him as a guide to more effective treatment, since they gave increased insight into the action of chemotherapeutic drugs. The chief phenomena of therapeutic biology are: (1) natural variations in therapeutic response determined by the host, according to species or individual and by the parasite according to species or strain; (2) reactions of acquired immunity on the part of the host and alterations in antigenic constitution of the parasite; (3) acquired drug-resistance of the parasite; and (4) therapeutic interference. All of these statements except the last one were published in their main features as conclusions reached in Ehrlich's laboratories. The original observations on which they are based will be outlined, and later novel developments will be mentioned.

Natural Variations in Therapeutic Response

Host. It was soon established that different species vary in their therapeutic responses; an interesting example is the greater effect of parafuchsin on *Trypanosoma brucei* in rats than in mice. Usually the higher susceptibility of the larger hosts to drugs in relation to body weight restricts their therapeutic value. Also, within any species, certain individuals tolerate a drug less readily than others.

Parasite. The powerful curative action of trypan red on *T. equinum* in mice is not obtained with *T. brucei* and several other species of trypanosomes, although all are similar in virulence. Different individual reactions to the same drug are shown by different strains, *e.g.* by *T. brucei* (Ehrlich's "ferox" and "debilis") or *T. congolense*; and when two drugs are tested with several strains of the same species the therapeutic responses are not necessarily parallel. It has been observed that a strain of *T. brucei*, when recently transferred to mice (therefore a strain of low virulence, as shown by the protracted course of the infection) gave a poorer response to various classes of drugs than the same strain when it had become more virulent following a further series of passages.[1] On the other hand, *e.g.*, in the case of two strains of *T. congolense*, both long established by acme passage (see below), each of statistically stable virulence, the therapeutic response of the less virulent was the greater.[2] With prolonged passages, some strains of high virulence have gained sensitiveness, *e.g.*, *T.*

rhodesiense for arsenicals. For this reason, among others, the use of "syringe-propagated" strains has sometimes been deprecated; but to abandon this type of strain would mean to neglect an invaluable tool. The real nature of such differences and the conditions which give rise to them are obscure.

Acquired Immunity of the Host and Alterations in Antigenic Constitution of the Parasite

With *T. brucei* and certain other species, repeated passage through small rodents leads to high virulence for these animals. When once parasites appear in the peripheral blood their number increases progressively, and death occurs in several days. Usually, a fairly large inoculum is used, as such an amount insures a constant, short incubation period. To preserve the strain, it is necessary to pass the infection to fresh animals as soon as trypanosomes are abundant in the blood. Such conditions minimize the chances of an immunity reaction of the host and this circumstance has the important effect of preserving the antigenic characters of the strain unchanged. But when the infection is sterilized by treatment, immunity occurs; it may be demonstrated on reinoculation with the same strain by failure of the host to become infected, *e.g.*, with Prowazek's *T. brucei*, but usually infection develops after a prolonged incubation period. The serum of cured animals contains antibodies formed against the original strain, which possess anti-infective properties, *etc.* When, after cure, reinoculation with the original strain leads to infection, the parasites are resistant to antibodies present in the serum, as Franke found. Similarly, after treatment with a suitable noncurative dose of drug, although the parasites disappear temporarily from the blood, they eventually reappear and are then also antibody-resistant. When a relapse (*i.e.* antibody-resistant) strain is transferred to fresh mice, these animals on cure develop immunity to reinoculation with this relapse strain, but not to the original strain. Thus the antigens (nutriceptors) of the relapse strain differ from those of the original strain; and when a succession of relapses occurs, a series of strains with different antigens are produced. The number of antigenic transformations so evoked tends to be more or less limited according to the species of host, *etc.*; sometimes the original antigen reappears; again, a strain, instead of possessing a single type of antigen (unio), may have two types (binio). Roehl and Gulbransen produced a relapse strain by treating trypanosomes briefly *in vitro* with serum containing the corresponding antibodies. Provided the antiserum is not present in excess, the treated trypanosomes on inoculation will cause infection and a relapse strain emerges. Ehrlich attributed this result to the antibodies blocking the nutriceptors of the trypanosomes, which consequently were forced to develop a fresh (relapse) type if they were to survive and multiply—an athreptic phenomenon. The factors involved must be highly complex.

Infection with *Trypanosoma brucei* or its allies in rabbits and larger animals follows a subacute or chronic relapsing course. Under these circumstances, there is a continuous antigenic flux. Similar behavior occurs in mice infected with less virulent trypanosomes, *e.g.*, *T. congolense*.[3] Here, in order to maintain a strain of constant antigenic constitution, it is essential to pass the infection by a fairly heavy inoculation of fresh animals as soon as parasites become abundant in the blood for the first time, *i.e.*, at acme.[2, 4] It is noteworthy that, after a certain submaximal degree of virulence has been reached, as compared with *T. brucei*, further acme passages over years do not cause any further increase; also different strains preserve statistically their individual behavior.

The antigenic lability of *T. congolense* is important practically in the therapy of experimental infections, because in a chronically infected animal, or in fresh mice to which a "chronic" strain (derived from such a mouse) has been transferred, the infection is less readily cured than an infection caused by the original acme strain. The relapse antigen of *T. congolense* may be an addition to the original acme strain antigen, not a replacement of it (Calver).[5] On repeated acme passage, the strain reverts more or less rapidly to the original type. One acme strain had a remarkable property: when mice cured of it were reinoculated repeatedly with the same strain, on each occasion 50 per cent of the immunes became infected. The experience of treatment of *T. congolense* in the field has not so far been analyzed in light of the above observations; consequently the supposed rapid development in treated cattle of drug-resistant strains may be erroneous. To decide the question it would be necessary to inoculate cattle with a mouse-adapted strain in the hope that it would preserve full mouse-virulence in relapses, so that, on returning the strain to the rodents, fallacies might not occur through estimating the therapeutic response during the period of reaccommodation (see above).

In small rodents, even when infection is scanty, sterilization by chemotherapy produces immunity more effectively than injections of dead trypanosomes, *e.g.*, *T. brucei* disrupted by water. The problem remains, however, why in many species of hosts subacute or chronic infections seldom or never undergo cure as the result of the spontaneous immunity reaction, but require, in addition, the administration of trypanocidal drugs.

Acquired Drug-resistance of Trypanosomes—Drug-fast Strains

Acquired drug-resistance was discovered as the result of attempting to treat repeated relapses and examining the reason for ultimate failure. This method of procedure appears to have been followed by Franke and then by Roehl, using parafuchsin in mice infected with *T. brucei*. For convenience, the drug was administered by feeding. The fact of prime importance is that, when the infection ceased altogether to respond to treatment, its transference to fresh mice produced fully virulent infections which also resisted parafuchsin. In other words, the trypanosomes had become drug-resistant independently of any influence of the individual host on the therapeutic effect. Similarly, strains resistant respectively to atoxyl and to trypan blue were then obtained. Of course, these drug-fast trypanosomes were also relapse strains. It was ascertained later that drug-fastness can be developed without altering the antigenic characters, by subjecting the parasites *in vivo*, in repeated passages, to increasing concentrations of drug, each too small to produce a therapeutic effect;[6] or, again, contact with the drug may occur *in vitro*, the treated organisms then being inoculated into mice. The rate of acquisition of resistance varies greatly, *ceteris paribus*, according to the particular drug and its chemical class. In difficult cases, *e.g.*, with suramin, success may be achieved by the aid of intravenous injection of colloidal copper, supposed to act by blocking cells of the RE system, or by very prolonged treatment; or the use of another drug related *qua* resistance may be effective. Acquired drug-resistance withstands cyclical transfer and may persist in animal passages even for periods of years without further contact with the drug. It will not be argued here whether drug-fastness is a mutation, an adaptation, or a continuing ("Dauer") modification. No certain means of abolishing it at will has been discovered, although Ehrlich stated that combined treatment inhibited the development of resistance; and some drugs, *e.g.*, suramin, have been considered to be "antimutative." Sometimes relapse strains have lost drug-resistance. Oesterlin[7] found that treatment with potassium hexatantalate *in vivo* restored sensitiveness to a strain fast to arsenicals.

It was established originally that acquired resistance to parafuchsin involved no increase in resistance to atoxyl or trypan blue, and that this effect is true also of strains fast to the latter drugs, but that, by successive treatments with the three compounds, a strain could be made fast to them all. A parafuchsin-fast strain is resistant to certain other triphenylmethane dyes; resistance to the aromatic arsenical drug atoxyl connotes fastness to various atoxyl derivatives ("arsenic-resistance" is a misnomer); and a trypan blue-fast strain resists trypan red, and also suramin, and *vice versa*. In the last instance, the chemical similarity of the compounds is not of a very close kind. The independence of resistance between these classes of drugs was the basis of Ehrlich's "therapeutic sieve." Even more arresting was the fact that resistance to trypaflavin (diaminoacridine derivative) or to other orthoquinonoid dyes and to the atoxyl class was mutual. Acquired drug-resistance has been used for analyzing the characters of chemoceptors, and more will be said of this procedure later. In the case of the pathogenic trypanosomes, practically no indications exist that insusceptibility to a drug may arise from their acquiring power to detoxicate the trypanocide. It must be added that this summary is a general outline from which many interesting details have been omitted.[8] Drug-resistance has been applied in genetic study: a mixture of two drug-resistant strains derived from the same original *T. brucei* was propagated in mice over repeated passages without causing any fusion of characters.(Browning).

Drug-resistance is a serious complication of chemotherapy. But it is not an invariable consequence of repeated treatment. Schnitzer[8] noted that unsuitable dosage with inadequate amounts of drug may lead to supersensitiveness of the strain. It has also been shown that the immunity reaction involved in the relapse procedure hinders the development of drug-resistance. With *T. congolense* in mice, the treatment of repeated relapses by the same dose of a phenanthridine drug resulted in the cure of as great a proportion of animals in the long run (up to a 20th relapse) as were cured initially by the same single dose.[2] On the therapeutic-sieve principle, Ehrlich advocated combined treatment with different classes of drugs, which had proved effective when Laveran originally used trypan red and arsenious acid to cure infections which could not be cured by either drug alone. However, combined treatment, although established experimentally in certain cases, has not been adopted to any extent practically. Suramin and pentamidine are unique, since the former inhibits the latter's toxicity without impaired therapeutic effect (Guimaraes and Lourie[9]).

It has been repeatedly recorded that, in the course of time, laboratory strains propagated by passage in untreated animals have become resistant spontaneously to some drug. Eagle and Magnuson[10] described such an instance in the case of amino–and amide–phenylarsenoxides and stated that contact with traces of drug could be practically excluded. This proviso, of course, is the crux of the matter. An observation of doubtful significance is that, in areas where treatment of human trypanosomiasis with arsenicals has long been practised, new cases show a poorer response than they have shown previously. The explanation may be either development of drug-fast strains or elimination of susceptible strains.[11]

Therapeutic Interference

This phenomenon was originally demonstrated as follows: a parafuchsin-fast strain of *T. brucei* in normal mice responds to an injection of trypaflavin just as the original strain does, but when parafuchsin is added to the animals' diet no therapeutic effect results. The paradoxical outcome of the combined action of the two trypanocidal agents was demonstrated before Ehrlich in 1907 by Miss R. Gulbransen and the writer. Since no published reference to the phenomenon was made by Ehrlich during his lifetime, the experiments were later repeated and put on record.[12] Schnitzer *et al.*[13] extended the observations and showed that a small dose of parafuchsin interferes with the action of trypaflavin on the normal strain, and that a more minute amount is effective with the parafuchsin-resistant strain. Further, the optimum effect is got by allowing an interval between injections of the two dyes—about four hours. Parafuchsin also interferes with arsacetin (the atoxyl class) and with salvarsan, but not with arsenophenylglycine. Methyl- and ethyl-violet, closely related to parafuchsin, also interfere, and trypan blue has a slight interfering action. A preliminary dose of parafuchsin itself does not interfere with a subsequent injection of a therapeutic dose of parafuchsin. In Schnitzer's "divided interference" experiment, parafuchsin is administered to an infected animal; then after some hours, its blood is used to inoculate a fresh mouse and, on treating the latter with trypaflavin, interference occurs. There can accordingly be little doubt that interference takes place on or in the body of the trypanosomes. The interfering effect of a dose passes off. Parafuchsin does not reduce the toxicity for the host of the substances with which it interferes, neither do mixtures show obvious precipitation or other changes. Recently Albert[14] has suggested that parafuchsin interferes with trypaflavin through mixed micelle formation.

Other investigators have since observed different antagonists of trypanocidal action.[8] Morgenroth and Rosenthal, for example, have recorded that potassium hexatantalate, although not obviously trypanocidal even in the highest dose, antagonized tartar emetic, while Voegtlin *et al.* experimentally supported Ehrlich's suggestion that the effect of arsenic was related to interaction with thiol (sulphydryl) groups of the protoplasm. They showed that reduced glutathione neutralizes the trypanocidal action of trivalent arsenicals both *in vivo* and *in vitro*. BAL, of course, is in this class. These facts will be discussed in relation to the mode of action of trypanocidal drugs. The list of interfering substances for certain trivalent arsenicals includes oxidation-reduction indicators having an appropriate potential, *p*-aminobenzoic acid, and ascorbic acid, the last drug interfering also with trypaflavin. It was suggested by Jancsó[15] that the oxidation-reduction indicators act by functioning as auxiliary respiratory catalysts. Schleyer and Schnitzer *et al.*[15] obtained interference with mapharside (3-NH_2:4-OH-phenylarsenoxide) and trypaflavin by means of esters or amides of substituted benzoic acids, *e.g.*, methyl-*p*-hydroxybenzoate, but the toxicity of the arsenical for the host was unaffected. The work of Williamson and Lourie[16] indicates that, in certain cases, there is a structural similarity between the interfering agent and the drug. Thus, against the arsenicals butarsen, mapharside, and melarsen oxide, *p*-aminobenzoic acid interferes with butarsen only (both have a *p*-COOH group); melamine and surfen C interfere only with melarsen oxide (all three have a *p*-melamine group); while glutathione, no doubt by virtue of its SH group, interferes with all three drugs. There is no constant parallelism between the effect of a compound in interfering with trypanocidal action and in neutralizing toxicity for the host. The mechanisms of the two phenomena, accordingly, can not be identical. Whether the various agents produce interference in one or several ways is unknown. The latter case appears more likely. It is of interest that a form of biological "interference" has attained importance in the action of viruses.

Interference is a theoretical risk of combined treatment. Also, a practical risk of fallacy arises in the examination of impure substances for trypanocidal action, since the product of a chemical side reaction in the preparation may mask the therapeutic effect of a more powerful constituent. On the other hand, new trypanocides have been discovered by tracing the activity of impure products to constituents derived from side reactions.

Trypanocidal Drugs, Their Constitution and Mechanism of Action

The *T. brucei* group, which was originally investigated, is sensitive to certain cotton-substantive bis-azo dyes (trypan red and trypan blue), which also are "vital" stains, and to more or less related compounds (especially suramin); dyes of the triphenylmethane group (especially parafuchsin); diaminostyrylquinoline derivatives and various other basic dyes; diamidines, quinine alkaloids, antrycide, furacin, achromycin, and some other organic compounds; metalloids, especially aromatic derivatives of arsenic and antimonials; bismuth salts, *etc.*[17] *T. congolense* is relatively insensitive even to the most active substances in the above list except to tryparosan (related to parafuchsin), to antimonials, and to certain aromatic polyarsenicals.[18] It responds also to surfen C, to dimethylstilbamidine, and to an aminocinnolylguanidine derivative ("528"), but especially to certain phenanthridine compounds and to antrycide.[18] *T. vivax* appears to be similar to *T. congolense* in its responses.

T. cruzi, so far, responds considerably only to Jensch's product (Bayer 7602 Ac) and to several phenanthridine urethano-compounds, which need not be specially active against *T. congolense*.[19] The death of intracellular forms has been noted in tissue cultures exposed to one of these drugs, no obvious damage to host cells being observed. Few compounds have found extended use for treating infections in the natural hosts. In man, the following may be mentioned for *T. gambiense*: arsenicals (atoxyl and, for the late stage when the central nervous system has become infected, tryparsamide, because of its significant capacity for passing the "blood-brain barrier"); and suramin and pentamidine, before involvement of the nervous system and for mass prophylaxis. *T. rhodesiense* responds to the same drugs, except arsenicals. In horses and cattle infected with the *T. brucei* group, including *T. evansi*: suramin and antrycide; in cattle infected with *T. congolense* and *T. vivax*: antimonials, surfen C and, especially, members of the phenanthridine series, and antrycide. It is uncertain how far the best drugs for clinical and field trials have been selected, since, almost inevitably, they were chosen on the basis of a high therapeutic index in laboratory animals, and then those drugs which appeared too toxic for the hosts were discarded.[20] While, in general, large animals are more liable to toxic effects than small ones, this generalization is not a universal rule. Also, special toxicity for particular species may be manifested quite unpredictably. Severe local tissue damage on subcutaneous or intramuscular injection of many substances is still an almost uncontrollable complication.

Apart from damaging the kidney or liver or producing supersensitiveness, trypanocidal agents have contributed some striking phenomena to therapeutic pathology: the production of "dancing" in mice by the neurotoxic action of arsacetin and similar atoxyl-derivatives, and optic atrophy in man, produced by atoxyl and tryparsamide; selective damage to the pancreatic islet cells produced by a diaminostyrylquinoline derivative; development of sarcoma from the phagocytic tissue produced in mice and rats by one subcutaneous injection of an aqueous solution of certain depot-forming diaminostyrylquinoline compounds (styryl "430" etc); and the still obscure, so-called photosensitization arising in cattle after treatment with a phenanthridine compound, especially in certain localities. This latter reaction is attributed to liver damage, and is not reproducible in laboratory rodents.

The sterilization of infection by a trypanocidal agent is probably seldom purely "internal disinfection," but is significantly contributed to by defense mechanisms of the host. Conclusive evidence of this belief was furnished by the experiments of Schnitzer and Silberstein,[21] in which mice were first inoculated with a normal strain of *T. brucei*, then, after one or two days, were reinoculated with a serologically identical derived strain which had been rendered fast to an arsenical drug and, finally, were cured by the arsenical. This result could have been achieved only through destruction of the drug-fast trypanosomes by antibodies developed in response to the parasites of the normal strain killed by the drug. Whenever the disappearance of infection is protracted (certain drugs, *e.g.*, suramin and phenanthridines, are slow in action) the operation of acquired immunity in conducing to cure cannot be excluded. Probably this result accounts for the fact that phenanthridine drugs cure *T. congolense* infection in mice more readily at acme than earlier. On the other hand, *T. brucei* infections are, in general, most easily sterilized at an early stage.

Ehrlich emphasized the aim of securing drugs with maximal parasitotropic and minimal organotropic properties. This maxim sums up the facts that, in the body of an infected animal, a drug trypanocidal *per se* or in the form of its metabolite, is subject to distribution, involving storage or excretion, and to chemical reaction, all of which may aid or impede access to the parasite and the effect upon it, and that, by toxic action on the host, the defensive mechanisms of the latter may be damaged. Apart from the actual mass administered, the route, the rate, and the volume of the dose may each, on occasion, be a significant factor in chemotherapy. When the balance of effects is sufficiently favorable, the host is cured and the parasites destroyed. Studies on relations between chemical constitution and therapeutic action record the end result of this complex interplay of forces, and some striking examples of it will be quoted. When a series of related compounds is examined, useful conclusions may be reached for the synthesis of still more effective members. But the results need not be valid for a different class. Acetylation affords an instance of successful analogy; arsacetin, the acetyl derivative of atoxyl, exerts markedly enhanced trypanocidal action in mice as compared with the parent atoxyl, largely because its lower toxicity permits higher dosage. In the diaminostyrylquinoline series, acetylation of one of the two amino groups usually has a similar effect.[17] Again, a low rate of excretion tends, within limits, to enhance trypanocidal action and to promote prophylactic properties. It appears that, in many instances, the bulk of a dose of effective drug is lost by too rapid removal from the body. However, as Eagle *et al.*[10, 22] have shown for a series of arsenoxides, toxicity for the host and trypanocidal action vary directly with the affinity of the drug for red corpuscles and parasites respectively, while toxicity varies inversely with the rate of excretion. In the case of suramin, slow excretion results from its fixation by body proteins with subsequent gradual liberation.[23] On the other hand, when injected into the tissues, a drug may precipitate, so forming a depot from which there is slow solution and absorption. An outstanding example is the water-soluble styryl-245;[24] with the lymph it produces locally a sparingly soluble complex which persists for many months, and the prophylactic effect may last for about a year, whereas a dose of a rapidly excreted congener ceases to protect after four days. The difference between the marked prophylactic effect of the sparingly soluble antrycide chloride, as compared with that of the much more soluble methylsulphate, seems to have this basis also.[25]

Since the introduction of trypaflavin (2:8-diamino-1·N-methylacridinium chloride) quaternization has been recognized as enhancing the trypanocidal action of certain N-containing basic compounds. The chemotherapeutic index of proflavine, the corresponding hydrochloride, may be less than one thirtieth of that of trypaflavin. Quaternization has a similar effect in the phenanthridine and cinnoline series. The quaternizing group has usually been CH_3, but C_2H_5 may sometimes be more advantageous (Watkins and Woolfe).[26] Albert[27] has suggested that the actively trypanocidal quaternary compounds may resist processes in the body which destroy or eliminate the corresponding nonquaternary substances. But not all quaternary aminoacridines are actively trypanocidal and this fact may be related to the stability of the tetracovalent N, which is high in trypaflavin. It is of interest in this connection that proflavine and trypaflavin (acriflavine) are powerfully and practically equally bactericidal *in vitro* for staphylococci.

Suramin (Bayer 205, moranyl) is impressive as an exceedingly potent trypanocide in mice, while its close chemical relatives are insignificant. The elucidation of its structure among a large group of possible compounds by Fourneau *et al.*[28] was an exciting piece of chemical detection and illustrates the help of good fortune in crowning an effort in scientific investigation. Removal of two methyl groups from the large suramin molecule practically abolishes the trypanocidal effect, and this result underlines the point that, so far, spatial relations and physical-chemical properties generally have been too little regarded in connection with the structure of trypanocides. Roehl[29] suggested that the trypanocidal action of suramin may be explained by its power of precipitating basic protein constituents of the trypanosomes.

In order to gain insight into the mode of action of trypanocidal drugs, their effects have been studied on the vitality of the parasites *in vitro*. Difficulties are encountered here, because motility (which may depend on acetylcholine[30]) and infectivity are not necessarily affected in parallel; and, of course, many substances act in the test tube which lack chemotherapeutic properties *e.g.*, mercuric chloride or phenol. Accordingly, such observations are chiefly of value in comparing series of compounds, some of which are powerful trypanocides *in vivo*. Biochemical methods are also used, *e.g.*, respiration and glucose-utilization by suspensions of trypanosomes, as a measure of their metabolic activity. Morphological changes may be difficult to interpret, since trypanosomes are exceedingly labile. However, permanent suppression of the blepharoplast by orthoquinonoid drugs is characteristic, although it apparently has no significance as regards other characters of the trypanosome, apart from arising concurrently with drug-fastness. But the blepharoplast may also disappear spontaneously. Irreversible accumulation of basophile granules in the cytoplasm of the parasites in infected animals under the influence of treatment has been interpreted by Ormerod[31] as evidence of interference with the normal cytoplasmic interaction of protein and ribonucleic acid. This effect is attributed to the molecules of the drugs bearing a permanent charge, so that they are retained in the trypanosomes. Again, while cytoplasmic nucleoprotein of trypanosomes sensitive to antrycide reacts with the drug and is split, acquisition of resistance involves loss of this reactivity. A very striking and fundamental observation made by Roehl and Gulbransen was that certain trypanocidal dyes, *e.g.*, triaminophenazoxonium chloride, *in vivo* quickly penetrate and stain the cytoplasm of a normal susceptible strain, whereas strains resistant to the dyes are colored only slowly and weakly. Other methods of demonstrating the same phenomenon consist in causing the drug-laden parasites to fluoresce under ultraviolet light or to be immobilized by exposure to brilliant white light. Also, susceptible parasites suspended in a solution of a trypanocidal dye or arsenical can be proved either to fix the drug or to deprive the fluid of it to an extent exceeding that of the same strain which has acquired resistance to the substance.[32]

Trypan red and suramin both illustrate a difficulty encountered in explaining trypanocidal action *in vivo* in terms of the behavior of mixtures of drug and parasites *in vitro*. Treatment in the test tube with a concentrated solution of trypan red neither immobilizes the trypanosomes nor affects their virulence on inoculation. Suramin under conditions *in vitro* which permit long survival of the parasites in absence of the drug, leads slowly to loss of virulence. On the other hand, parafuchsin *in vitro* quickly immobilizes in concentrations which fail to abolish the power of multiplication *in vivo*. Antrycide [4-amino-6-(2′-amino-1′:6′-dimethylpyridinium-4′-amino)-1:2-dimethylquinolinium dichloride or dimethylsulphate], which acts on the *T. brucei* group, but especially on *T. congolense*, is also puzzling. *In vitro* a high concentration does not kill *T. equiperdum* or affect respiration and glucose-metabolism over a long period (20 hours), but *in vivo* a therapeutic dose in 24 hours renders the parasites noninfective for fresh mice; nevertheless the parasites persist in the blood of the treated animal for a day or longer, although gradually ceasing to divide. It remains undetermined whether the curative effect is owing to slow penetration and action within the cell rather than on the surface, the cytoplasm being primarily affected, or whether growth is inhibited rather than the cell killed by the drug (Ormerod[31]). The possibility that a decisive effect may be attributed to interference with growth *per se* is demonstrated by the failure of *T. congolense* to infect mice fed on a diet in which casein is the sole source of protein (Keppie[33]). This work suggests a fresh approach in dealing with trypanosomiasis where diet can be controlled, as in man. The discovery of the trypanocidal diamidines followed the finding that synthalin had some action. The latter was tried on the supposition that hypoglycemia would adversely affect the vitality of the trypanosomes by starving them of sugar, but a similar hypoglycemia produced by insulin or guanidine has no therapeutic effects; also synthalin is highly trypanocidal *in vitro*.[34] King *et al.* then synthesized various active aliphatic and aromatic diamidines and related compounds and finally Ewins *et al.* prepared stilbamidine, propamidine, and pentamidine.[35]

Arsenicals will be dealt with in more detail, because there were great synthetic possibilities in this series, and their therapeutic action on both trypanosome and spirochaete infections made them specially important. Thomas originally noted the value of atoxyl in trypanosomiasis, Ehrlich having discarded it owing to his strain being insensitive. The constitution of an anilide currently attributed to the drug was discouraging for further chemical developments. But there can be little doubt that Ehrlich tested its chemical reactivity, as was his wont, and found that it could be diazotized. With Bertheim's aid, it was identified as the sodium salt of *p*-aminophenylarsonic (arsanilic) acid, a highly reactive substance containing pentavalent arsenic. From this start, several thousand organic compounds of arsenic have now been prepared with a view to obtaining powerful chemotherapeutic agents. It can not be asserted that the trove is exhausted yet, since among recent products, Eagle's butarsen[36] is of proved efficacy and Friedheim's melamine derivatives,[37] as well as acting on atoxyl-fast trypanosomes, promise high value at all stages of *T. gambiense* infection in man, while a similar antimony compound has great prophylactic action.

Atoxyl and related compounds (*e.g.*, tryparsamide) in proportion to their arsenic content are very slightly toxic for mice, *etc.*, and almost harmless to trypanosomes *in vitro*. The corresponding trivalent reduction products, arsenoxides (arsenosobenzenes), are highly toxic and rapidly trypanocidal both *in vivo* and *in vitro*. The arsenobenzenes are intermediate. There were indications also that the mice least tolerant of atoxyl were those in which the best trypanocidal response resulted. It was accordingly deduced that the active compound is the arsenoxide, into which the other two classes are converted in the living body, but only recently has this deduction been substantiated chemically for a pentavalent compound.[38] Ehrlich decided, on the evidence before him, that the arsenobenzenes were the best drugs for practical therapy. They lacked the pronounced neurotoxic action of arsonic acids and the high general toxicity of arsenoxides. Afterwards, the advantage of one of the latter (mapharside)[39], corresponding to salvarsan, was seen in its stability and in its freedom from liability to more toxic admixtures, and also in its having a curative index similar to that of the arsenobenzene. Two arsenobenzenes were chosen originally: arsenophenylglycine for its high activity against *T. brucei* in mice and rabbits, which unfortunately was not realized in larger animals; and salvarsan or arsphenamine (3:3′-diamino-4:4′-dioxyarsenobenzene) for its potency against trypanosomes and especially spirochaetes. In default of reliable chemical criteria of purity, the action of salvarsan on *Trypanosoma equiperdum* in mice was adopted as a reliable biological control of its therapeutic action in *Tr. pallidum* infection in humans.

Ehrlich interpreted the development of drug-resistance as owing to a reduction in affinity of the chemoceptors for the drug, and ample experimental evidence supports this deduction. A striking property of arsenophenylglycine is its ability to influence strains of trypanosomes resistant to the atoxyl compounds. Such a strain could be rendered fast to arsenophenylglycine, however, by treatment with this drug. Therefore, Ehrlich concluded that it had a greater affinity for the trypanosomes and possessed an extra haptophore group, the aceticoceptor. The work of King[40] at length supplied a satisfactory physical-chemical explanation of the results. Having noted an earlier observation that atoxyl-resistant trypanosomes were as susceptible to phenylglycinearsonic acid as the normal strain, he compared the effects *in vitro* of a large series of different arsenoxides on normal and atoxyl-fast strains. These drugs fell more or less into three classes: (1) those having a free carboxyl group, which form neutral sodium salts with a high affinity for water (ionized in solution) and are equally toxic for the normal and the resistant strains—the arsenoxide corresponding to arsenophenylglycine and butarsen (γ-(p-arsenosophenyl) -butyric acid) belong to this class; (2) those which, like phenylarsenoxide and xylylarsenoxide, lack hydrophilic groups apart from the AsO group and therefore, at a lipoid-water interface, would travel with the phenyl or xylyl group in the lipoid and the AsO in the water (it should be noted that trypanosomes are rich in lipoids) and act very powerfully and equally on both strains; and (3) those which, like the arsenoxides of atoxyl and arsacetin, act 30 to 60 times more intensely on the normal than the resistant strain. King suggests that compounds of the third class are substantive for the same chemically polar constituents of the parasites as fix trypaflavin (since it has been shown that strains rendered fast to this dye and to arsacetin respectively are identical in their properties), while, on the other hand, parafuchsin and trypan blue are fixed by other constituents. It is of interest that phenyl-*p*:*p*′-diarsenoxide belongs to the third class. There is no evidence that substances of the second class have, in general, a greater chemical reactivity than those of the third class. Accordingly, King does not consider that the final chemical mechanism of the action of arsenical trypanocides has been discovered, but merely their first phase of action.

The interference effect is also consistent with a chemoceptor mechanism. Difficulties arise, however, on attempting to align the phenomena with those of acquired resistance. Schnitzer[13] used interference as a method of gaining biological knowledge of the arsenical chemoceptor to amplify the information derived from the so-called arsenic-resistant strains. The investigation, which yielded a complex picture, may be outlined as follows: interference is not an aspect of resistance, since there is no necessary parallelism between the capacity of one compound to interfere with another and to produce resistance to the latter. Also, repeated interference does not produce any change in the drug-sensitiveness of the strain of trypanosomes operated on. The conclusion is reached that the arsenical chemoceptor or center (*viz.*, that related to the atoxyl and salvarsan classes of compounds *etc.*) is composed of several primary fixation centers of relatively low specificity, dominated by the respective interfering compounds parafuchsin, sulphydryl, and tantalate, with highly specific separate secondary centers for fixing drugs of the atoxyl class (arsacetin *etc.*), salvarsan, trypaflavin, and antimony. It is inferred that the affinity of a trypanocidal drug for a particular center is inversely proportional to the ease with which its action can be interfered with; *e.g.*, in the case of parafuchsin-fast *T. brucei*, tartar emetic has a low affinity for the parafuchsin center, since even a large dose of parafuchsin scarcely produces interference.

At present, for the explanation of phenomena such as chemotherapeutic effects, attention is centered on disturbances of the action of enzymes, which maintain a chain of syntheses of substances essential for the growth of the parasites, such disturbances being held responsible for those effects. An approach to chemotherapy which has been proposed as "rational" by Fildes[41] is to ascertain the nature of those essential substances and then to devise drugs which are modifications of them but are endowed with the property of interrupting the chain. This interruption may be effected by perverting some reaction (as by leading to an oxidation instead of a reduction), or by producing an inactive product through molecular combination, or by competing for an enzyme associated with an essential substance. In considering such projects, it would be well to recall the statement of King that "with a complete knowledge of the structure and of all the reactions going on in the trypanosome-cell and of the enzymes involved, it should be possible to be certain of the final mechanism of an arsenical, but this state of knowledge is impossible to attain without the aid of Maxwell's sorting demons."

Ehrlich's experimental facts have stood the test of time and have formed the basis of a new branch of science as well as of a flourishing industry. His theories, which provided a logical and attractive setting for the facts, rendered the subject interesting and understandable to others less deeply versed than himself and, as in the case of the receptor theory of cellular nutrition, proved the inspired anticipation of a truth. Ehrlich has been criticized for oversimplification; but in his own words, "a glimpse into life can be gained only from living matter itself, and one must realize how little of it we know and how little we shall know." So it can be taken as axiomatic that he never was deceived into the belief that the current structural symbolism of the chemists fully portrayed the complexity of the vital processes. Forty years after Ehrlich, the chemoceptor may be more aptly compared with an organ such as the hand than it may be expressed by a chemical formula, and it is plain that much further knowledge must accumulate before the biological and the physical-chemical outlooks can converge to a focus.

Bibliography

Complete references to the publications of Ehrlich and his co-workers are contained in "Paul Ehrlich" (Jena, 1914). A summary of the literature to 1930 is given in the writer's article on "Chemotherapy," Medical Research Council's *System of Bacteriology* 1931, **6:** 501 (London).

1. Browning, C. H. & R. Gulbransen. 1935. J. Hyg. **35:** 180.
2. Browning, C. H. & K. M. Calver. 1943. J. Path. Bact. **55:** 393.
3. Browning, C. H., H. Adamson & A. A. N. Keppie. 1953. J. Path. Bact. **65:** 137.
4. Browning, C. H., K. M. Calver & H. Adamson. 1948. J. Path. Bact. **60:** 336.
5. Calver, K. M. 1945. Trop. Diseases Bull. **42:** 704.
6. Lewy, F. & M. Gurewitsch. 1926. Z. Hyg. Infektionskrankh. **106:** 532.
7. Oesterlin, M. 1941. Zentr. Bakt. Parasitenk. Ab. I. Orig. **147:** 335.
8. Schnitzer, R., 1932. Weichardts. Ergeb. Hyg. Bakt. Immunitätsforsch. Exptl. Therap. **13:** 227. R. J. Schnitzer, L. C. Laverty & M. Buck. 1946. J. Immunol. **54:** 47.
9. Guimaraes, J. L. & E. M. Lourie. 1951. Brit. J. Pharmacol. **6:** 514.
10. Eagle, H. & H. J. Magnuson. 1944. J. Pharmacol. Exptl. Therap. **82:** 137.
11. Van Hoof, L. M. J. J. 1946–47. Trans. Roy. Soc. Trop. Med. Hyg. **40:** 728.
12. Browning, C. H. & R. Gulbransen. 1922. J. Path. Bact. **25:** 395.
13. Schnitzer, R. *et al.* 1926. Z. Immunitätsforsch. **47:** 116; 1926. *Ibid.* **48:** 23; 1926–27. *Ibid.* **49:** 387, 393, 551; 1927. *Ibid.* **53:** 439; 1932. *Ibid.* **75:** 143; 1936. *Ibid.* **88:** 415. W. Silberstein. 1928. *Ibid.* **54:** 324.
14. Albert, A. 1949. Soc. Exptl. Biol. Rept. 3rd Symposium on Selective Toxicity *etc.*: 318. Cambridge, England.
15. Jancsó, N. & H. Jancsó. 1936. Z. Immunitätsforsch. **88:** 275. W. L. Schleyer & R. J. Schnitzer. 1948. J. Immunol. **60:** 265. R. J. Schnitzer & D. R. Kelly. 1950. *Ibid.* **64:** 95.
16. Williamson, J. & E. M. Lourie. 1948. Nature. **161:** 103.
17. Hewitt, R. I. *et al.* 1953. Am. J. Trop. Med. Hyg. **2:** 254 (achromycin). F. H. S Curd & D. G. Davey. 1950. Brit. J. Pharmacol. **5:** 25 (antrycide). E. M. Lourie & W. Yorke. 1939. Ann. Trop. Med. Parasitol. **33:** 289 (diamidines). C. H. Browning, J. B. Cohen, S. Ellingworth & R. Gulbransen. 1926. J. Path. Bact. **29:** 317; 1929. Proc. Roy. Soc. (B)**105:** 99 (diaminostyrylquinolines). J. C. Krantz, Jr. & W. E. Evans, Jr. 1945. J. Pharmacol. Exptl. Therap. **85:** 324 (furacin). N. J. Giarman. 1951. *Ibid.* **102:** 185 (furacin).
18. Davey, D. G. 1950. Trans. Roy. Soc. Trop. Med. Hyg. **43:** 583; see also Curd *et al.*[17] (antrycide). J. R. Keneford, *et al.* 1948. Nature. **161:** 603, 758; 1952. J. Chem. Soc. : 2595 (aminocinnolylguanidine). E. M. Lourie *et al.* 1951. Brit. J. Pharmacol. **6:** 643 (aminocinnolylguanidine). C. H. Browning, G. T. Morgan, J. V. M. Robb & L. P. Walls. 1938. J. Path. Bact. **46:** 203 (phenanthridines). C. H. Browning. 1949. Nature. **163:** 590 (phenanthridines). G. Brownlee *et al.* 1950. Brit. J.

Pharmacol. **5:** 261 (phenanthridines). E. Fourneau *et al.* 1933. Compt. rend. Acad. Sci. **196:** 1173 (polyarsenicals).

19. Jensch, H. 1937. Angew. Chem. **50:** 891. C. H. Browning, K. M. Calver, M. W. Leckie & L. P. Walls. 1946. Nature. **157:** 263 (phenanthridines). L. G. Goodwin, M. D. Goss, J. A. Lock & L. P. Walls. 1950. Brit. J. Pharmacol. **5:** 277. (phenanthridines). J. A. Lock. 1950. *Ibid.* **5:** 398 (phenanthridines—action on intracellular forms).
20. Lourie, E. M. 1952. The Analyst. **77:** 175.
21. Schnitzer, R. & W. Silberstein. 1928. Z. Immunitätsforsch. **58:** 159.
22. Hogan, R. B. & H. Eagle. 1944. J. Pharmacol. Exptl. Therap. **80:** 93. H. Eagle. 1945. *Ibid.* **85:** 265. H. Eagle & G. O. Doak. 1951. Pharmacol. Rev. **3:** 107.
23. Boursnell, J. C. & A. Wormall. 1939. Biochem. J. **33:** 1191. A. Spinks. 1948. *Ibid.* **42:** 109.
24. Browning, C. H. & R. Gulbransen. 1934. J. Path. Bact. **39:** 75.
25. Spinks, A. 1950. Brit. J. Pharmacol. **5:** 445.
26. Watkins, T. I. & G. Woolfe. 1952. Nature. **169:** 506.
27. Albert, A. 1951. The Acridines. : 231 *et seq.* London.
28. Fourneau, E., Mme. J. Tréfouel & J. Valée. 1924. Ann. inst. Pasteur. **38:** 81.
29. Roehl, W. 1926. Arch. Schiffs- u. Tropen-Hyg. **30** (Beihft. 1) :103.
30. Bülbring, E., E. M. Lourie & U. Pardoe. 1949. Brit. J. Pharmacol. **4:** 290.
31. Ormerod, W. E. 1951. Brit. J. Pharmacol. **6:** 325, 334; 1952. *Ibid.* **7:** 674. F. W. Schueler. 1947. J. Infectious Diseases. **81:** 139 (granules stainable at pH3 in mapharside-fast strain).
32. Jancsó, N. 1932. Zentr. Bakt., Parasitenk. Abt. I. Orig. **124:** 167 (optical methods). N. Jancsó. 1932. Klin. Wochschr. **11:** 689 (optical methods). F. Hawking. 1937. J. Pharmacol. Exptl. Therap. **59:** 123 (estimation of arsenicals).
33. Keppie, A. A. N. 1953. Brit. Med. J. **2:** 853.
34. Lourie, E. M. & W. Yorke 1937 Ann. Trop. Med. Parasitol. **31:** 435 (action of synthalin *in vitro*). P. Browning. 1938. J. Path. Bact. **46:** 323 (hypoglycaemia).
35. Lourie, E. M. & W. Yorke. 1939. Ann. Trop. Med. Parasitol. **33:** 289.
36. Eagle, H. 1945. Science. (n.s.) **101:** 69.
37. Friedheim, E. A. H. 1940. Ann. inst. Pasteur. **65:** 108.
38. Crawford, T. B. & G. A. Levvy. 1947. Biochem. J. **41:** 333.
39. Tatum, A. L. & G. A. Cooper. 1932. Science. **75:** 541; 1934. J. Pharmacol. Exptl. Therap. **50:** 198.
40. King, H. 1943. Trans. Faraday Soc. **39:** 383.
41. Fildes, P. 1940. Lancet. **1:** 955.

CHEMOTHERAPY OF SPIROCHETAL INFECTIONS

By John A. Kolmer

Institute for Public Health and Preventive Medicine, Temple University School of Medicine, Philadelphia, Pa.

Foreword

The New York Academy of Sciences is to be congratulated on holding this conference in memory of Paul Ehrlich, founder of chemotherapy, born on March 14, 1854. I wish to express deep appreciation of the opportunity of participating in such a conference, since the death of this great investigator from cerebral apoplexy on Aug. 20, 1915, was but little noticed by the world, which he served so well and unselfishly, because of the turmoil of World War I.

I had the honor and privilege of knowing Ehrlich personally. He kindly welcomed me, and permitted me to spend a period of time, during August 1912, in his Institute for Experimental Therapy in Frankfort, Germany. Nervous and almost inarticulate as I was upon meeting this great and busy man, I shall never forget his exuberance and innate kindness. As he spoke German in a rapid and peculiar manner, I had some difficulty at first in understanding him, but I soon regained sufficient composure to do so without exhausting his limited patience. As I remember, most of our conversation at that time was in relation to the administration and toxicity of salvarsan; he was particularly anxious to know if any serious or fatal reactions from the use of the product had occurred in Philadelphia. He inquired as to my lodgings, and after being assured that I had secured comfortable living quarters in Frankfort, he suddenly turned, rushed into the corridor of the Institute and, with cupped hands, shouted to his faithful factotum Kadereit, somewhere on an upper floor, "Kommen sie herunter." He introduced Kadereit to me and instructed him to take me through the Institute and the adjoining George Speyer-Haus. This tour of inspection gave me the opportunity of meeting some of Ehrlich's noted colleagues, among them the genial and industrious Hata, who at that time was working with selenium-eosin compounds in the treatment of experimental cancers of mice; the tall and handsome Hans Sachs, with whom I subsequently spent considerable time in discussions of the Wassermann test; and the faithful but reticent Alfred Bertheim. I particularly appreciated my contacts with Hata, from whom I learned the technic of conducting experimental tests for syphilis on rabbits, and trypanocidal tests on rats and mice, a knowledge which was to serve me well in 1914-1915, when Schamberg, Raiziss, and I succeeded in solving the intricate synthesis of salvarsan, designated as arsenobenzol, and later renamed arsphenamine. This development gave us the opportunity of supplying the compound for our Armed Forces, for the public health services, and for the country at large during the war, when supplies of the German product became exhausted and finally unobtainable. I shall never forget the frequent routine rounds of the Institute made by the indefatigable Ehrlich, his habit of always smoking big Havana cigars, his indifference to outward appearances, his excitable temperament with so much "wissen sie—verstehen sie?"; and, as told me by Hata, his four "g's" for successful research, namely: *geduld*, *geschick*, *geld*, and *glück* (patience, ability, money, and luck); and in so far as *geld* is concerned, it is now comforting to recollect that Ehrlich, through his friend Simon Flexner, obtained a substantial grant from John D. Rockefeller which aided him very materially in the synthesis of new arsenical compounds and their testing in the treatment of various experimental spirochetal infections by Hata, tests culminating in the synthesis of salvarsan.

Historical. The basic idea or concept dominating the entire life work of Ehrlich in the treatment of infectious diseases is to be found in his earliest publication, which appeared in 1877. He was then 23 years old, and had not yet completed his graduation in medicine.[1] Certainly, this basic concept of his relating to chemical agents, which he later designated as "chemotherapy," was apparent as early as 1891, when he observed that methylene blue possessed some value in the treatment of human malaria. Ehrlich's results in the chemotherapy of experimental tuberculosis and other infections, however, were so disappointing that he attempted nothing further in this field until 1905, when he became greatly interested and encouraged by a discovery made by Thomas,[2] who found that atoxyl was effective in the treatment of experimental trypanosomiasis. Undoubtedly this finding stimulated Ehrlich into starting the series of remarkable and laborious investigations[3] resulting in the synthesis of arsacetin, arsenophenylglycin, and numerous other spirocheticidal compounds, culminating, in 1907, in the synthesis of salvarsan or compound 606. Up to that time, only four specific remedies were known in the treatment of spirochetal and protozoal diseases, namely, mercury for syphilis, quinine for malaria, ipecac for amebic dysentery, and atoxyl for trypanosomiasis. *Treponema pallidum* was discovered by Schaudinn and Hoffmann in 1905; the complement fixation test for syphilis was formulated by Wassermann, Neisser, and Bruck and, independently by Detre, in 1906. Thus, within a period of only three years, the cause of syphilis, a serologic test of great value in its detection, and a sovereign remedy for its treatment were discovered, a triad of developments in the annals of this disease without parallel in the history of medicine.

Unfortunately, however, Ehrlich laid salvarsan aside in 1907, when one of his assistants reported to him that it was ineffective in the treatment of experimental trypanosomiasis. As related by his biographer, Miss Marquardt,[1] the reason for this action may never be known. Fortunately, however, Hata, upon joining Ehrlich's staff in 1909, promptly found that salvarsan was highly effective not only in the treatment of experimental trypanosomiasis, but also in the treatment of experimental relapsing fever in mice, experimental syphilis in rabbits, and spirochetosis in fowls. As a result, salvarsan was not employed in the treatment of human syphilis and relapsing fever until 1909, when Ehrlich reported the results of its use in this connection to the world at the Congress for Internal Medicine held at Wiesbaden on April 19, 1910. Consequently, Ehrlich's dream of "magic bullets" and *therapia sterilisans magna* (curing and healing with single large doses) was realized in these experimental infections.

At that time, Ehrlich also thought that single large doses of salvarsan might suffice for the complete or biologic cure of early syphilis, of relapsing fever, and of frambesia, or yaws, in human beings, and of pleurisy in horses. Unfortunately he did not live long enough to see this dream fulfilled as regards the treatment of syphilis in human beings, an accomplishment now thought possible by means of single large doses of penicillin administered by intramuscular injection. Greatly worried and annoyed by errors in the administration of salvarsan which had resulted in a high incidence of toxic and even some fatal reactions, Ehrlich nevertheless continued his investigations which, in 1912, resulted in the synthesis of neosalvarsan, or compound 914, a product of lesser toxicity and spirochetal activity. Under the circumstances, the discovery of salvarsan was not the first but the last major and, possibly, the greatest accomplishment of this investigator of blessed memory.

Needless to say, however, these remarkable discoveries resulted in a great deal of investigation conducted by other observers, resulting in the synthesis of such additional trivalent and pentavalent arsenical compounds as sodium salvarsan, silver arsphenamine, neoarsphenamine, sulpharsphenamine, mapharsen, tryparsamide, stovarsol, acetarsone, *etc.*, for the treatment of syphilis and other spirochetal and trypanosomal diseases. In 1916, Sauton and Robert found that sodium tartrobismuthate was also effective in the treatment of fowl spirillosis, which led to a discovery made by Sazerac and Levaditi, in 1920–1922, to the effect that potassium and sodium tartrobismuthate were so much more spirocheticidal and less toxic than mercury that such organic bismuth compounds, including bismarsen, soon replaced mercury, which had been used since 1497, in the treatment of syphilis. Finally, since the discovery of the remarkable therapeutic activity of penicillin in syphilis by Mahoney, Arnold, and Harris[4] in 1943, this antibiotic compound has not only entirely eliminated tryparsamide in the treatment of neurosyphilis, but has almost completely replaced arseno-bismuth therapy not only in the treatment of syphilis but of other treponemal, borreliar, and leptospiral infections as well.

Pathogenic Spirochetes

Three genera of Spirochaetacea are pathogenic for man, namely, *Treponema*, *Borrelia*, and *Leptospira* (TABLE 1). Bejel is commonly regarded as owing to

TABLE 1

PATHOGENIC SPIROCHETES

Treponema	*T. pallidum* (syphilis, bejel)
	T. pertenue (frambesia or yaws)
	T. carateum (pinta)
Borrelia	*Bor. recurrentis, novyi, duttonii, etc.* (relapsing fever)
	Bor. muris or *Spirillum minus* (rat-bite fever)
	Bor. vincentii (fusospirochetal infections)
Leptospira	*L. icterohemorrhagica* (infectious jaundice)
	L. canicola (infectious jaundice)
	L. hebdomadis (Japanese seven-day fever)
	L. grippo-typhosa (swamp fever)
	L. pomona (Pomona fever), *etc.*

infection with *T. pallidum*. Certainly the disease is difficult to distinguish from syphilis and is very closely related to, if not identical with this disease except in its nonvenereal transmission. Frambesia or yaws is now regarded as attributable to infection with *T. pertenue* although some investigators believe that this disease may be only a tropical type of syphilis. Many different species of *Borrelia* have been identified with the etiology of relapsing fever but not all of these have been clearly identified as separate species. Undoubtedly *Borrelia vincentii* is to be regarded as a pathogen in the production of Vincent's angina, trench mouth, tropical or phagedenic ulcer, *etc.*, in symbiosis with *Fusobacterium plauti-vincentii*. Likewise, in the etiology of pulmonary abscess and gangrene, *etc.*, although it is possible that relatively nonpathogenic species like *Borrelia buccale*, *B. microdentium* (*T. microdentium*), *B. macrodentium* (*T. macrodentium*) and other oral spirochetes may also play a role in the etiology of these infections, including bronchopulmonary spirochetosis. About eleven pathogenic species of *Leptospira* have been identified in the etiology of infectious jaundice (Weil's disease), Japanese seven-day fever, swamp fever, Pomona fever, and other leptospiral infections.

Syphilis

Penicillin. The fact that penicillin has almost entirely replaced arseno-bismuth compounds in the chemotherapy of syphilis is not owing, however, to higher treponemicidal activity, but to lower toxicity permitting the administration of larger doses. Indeed, as shown in TABLE 2, in my laboratory, the average single minimal curative doses of penicillin, in terms of mgm./kg. in the treatment of acute syphilitic orchitis of rabbits, have been 6 to 12 times larger than the average single minimal curative doses of arsphenamine, neoarsphenamine, mapharsen, and bismuth salicylate. Furthermore, I cannot refrain from referring also to the fact that the inorganic iodides, which have been employed in the treatment of syphilis since 1821, likewise have been almost entirely abandoned since the advent of penicillin therapy. It is true that the treponemicidal activity of these iodides is so low that they may be omitted in the treatment of early syphilis; but there can be no doubt of their value in the treatment of late-acquired and congenital syphilis, as they promote the resolution or absorption of microscopic and macroscopic gummatous lesions and syphilitic exudates, facilitating or promoting the penetration and

TABLE 2

AVERAGE SINGLE MINIMAL CURATIVE DOSES IN THE TREATMENT OF ACUTE SYPHILITIC ORCHITIS OF RABBITS

Compounds	Route of admin.	Mg. per kg.
Arsphenamine	Intraven.	10–12
Neoarsphenamine	"	15–20
Mapharsen	"	8–10
Bismuth salicylate	Intramus.	10–15
Crystalline penicillin G*	"	100–120

* 1624 units per mg.

therapeutic activity of more active treponemicidal compounds. In my laboratory, at least, the administration of both sodium and potassium iodides has been found to enhance the therapeutic activity not only of the trivalent arsenical compounds, bismuth salicylate, and mercury, but that of penicillin, as well, in the treatment of experimental syphilis in rabbits.[5, 6] Under the circumstances, I always administer potassium iodide orally in the penicillin treatment of latent, tertiary acquired, and late or tardive congenital syphilis, and I believe that this combination therapy deserves more attention at the present time.

As most observers well know, microcrystalline procaine penicillin G in aqueous and oil suspensions, especially in oil gelled with 2 per cent aluminum monosterate (PAM), is now being widely employed. I am very favorably impressed also by the therapeutic activity of Bicillin L-A (benzathine penicillin G) in aqueous suspension in the treatment of both experimental and human syphilis. Needless to state, the intramuscular injection of these repository penicillins has greatly simplified and aided the adequate treatment of syphilis with special reference to ambulatory cases of the disease.

Fortunately, the therapeutic activity of penicillin does not appear to be modified in an important degree by chemical transformation regardless of any binding action by body proteins and, while the absolute minimum blood concentration required in the treatment of syphilis is still unknown, it is commonly thought to be about 0.03 units/ml. of serum. However, it is practically certain that the magnitude, and particularly the duration, of penicillin concentration at foci of infection are of primary importance in treatment. Unfortunately, however, there are no methods available for determining these tissue concentrations, although it appears that blood levels roughly indicate the concentrations of penicillin acting in at least acute foci of infection. Furthermore, it appears that the therapeutic efficacy of different dosages and treatment schedules depend primarily on the *time* over which they provide effective concentrations of penicillin in the blood, and thus in the tissues, regardless of initially high but relatively brief blood levels. In the treatment of acute or early-acquired and congenital syphilis, it is also likely that relatively high blood concentrations over short periods are as therapeutically effective, or even more effective, than relatively low concentrations maintained over longer periods of time. In latent, in tertiary-acquired, and in late or tardive congenital syphilis, however, it is necessary to provide prolonged effective blood concentrations of penicillin in the blood in view of the time required for the penetration and building up of therapeutically effective concentrations of the compound in the sclerotic lesions of the disease. Fortunately, in the penicillin treatment of experimental syphilis of rabbits, it appears that therapeutic activity may continue for at least 12 to 24 hours beyond the time that the compound is demonstrable in the blood, which is probably also true in human syphilis.

Largely owing to the splendid investigations of the Clinical Cooperative Group, we have had fairly well-defined information in regard to the minimum amounts of arseno-bismuth therapy required for the complete or biologic cure of early, early-latent acquired, and early congenital syphilis as, likewise, for symptomatic and serologic "cures" of late-latent, late-acquired, and congenital syphilis. Except in the treatment of early acquired and congenital syphilis, however, it is still impossible to postulate on the total amount and administration of penicillin required in the treatment of latent, late-acquired, and late congenital syphilis, because the results reported have varied so much according to different schedules of treatment. Under the circumstances, it is greatly hoped that sooner or later a Clinical Cooperative Group will also tackle this problem and authoritatively establish minimum amounts of penicillin for the treatment of all types of acquired and congenital syphilis, subject to changes in individual cases of the disease.

At the present time it appears that single doses of 1.2 million units of PAM may suffice for the treatment of seronegative primary and 2.4 million units for seropositive primary and secondary syphilis, although clinical and/or serologic relapses may occur in about 3.0 to 15 per cent. This method of treatment is particularly valuable from the standpoint of public health in promptly destroying *T. pallidum* in the lesions and thereby reducing the incidence of infectiousness and transmission. On the other hand, however, while it is still difficult and frequently impossible to differentiate between clinical and/or serologic relapses and reinfections, it appears that, while the incidence of reinfections after adequate arseno-bismuth therapy was about 0.5 per cent, it may vary from about 3 to 18 per cent or more after penicillin therapy, owing to the higher percentages of biologic cures, which are higher in Negroes than in white individuals.

Apparently most cases of latent syphilis do not progress to the tertiary stage even in the absence of treatment. Penicillin is doubtless just as effective or more so than arseno-bismuth therapy in early-latent syphilis. The same is true in the treatment of late-latent syphilis, although it is still too soon to draw conclusions. Penicillin therapy, however, does not guarantee arrest of the disease. Certainly, progression occurs more frequently in seroresistant cases with the possibility that penicillin is less effective than arseno-bismuth therapy in securing serologic reversals.[7] As in arseno-bismuth therapy, re-treatment may be of but little additional benefit in cases previously given reasonably adequate treatment. The great problem is reaching a decision upon whether positive serologic reactions are attributable to latent syphilis or to biologic false positive reactions. If leprosy and malaria can be excluded, and if the TPI test cannot be conducted as an aid in differential diagnosis, it is my practice to institute penicillin treatment in order to "play safe," especially if positive serologic reactions persist for at least four to six months, since "acute" biologic false positive reactions are always likely to undergo spontaneous reversal within this time.

As in arseno-bismuth therapy, the penicillin treatment of seroresistant or "Wassermann-fast" cases of syphilis continues to be the bugbear of syphilotherapy. Seroresistance may be considered present in cases showing persistently positive serologic reactions six to twenty-four months following adequate initial treatment. In early and early-latent acquired syphilis, adequately treated with arseno-bismuth compounds, seroresistance occurred in from 0.2 to 12 per cent of cases and in from 5 to 20 per cent of cases of early congenital syphilis. While the incidence in early acquired syphilis adequately treated with penicillin cannot be stated, it is certainly very low and much lower than observed in cases treated with arseno-bismuth compounds. The cause of seroresistance is still unknown. But since I have observed that the reagin disappears from the blood of rabbits having acute syphilitic orchitis within 15 weeks after biologic cure, and in about six months after biologic cure in the case of rabbits afflicted with chronic syphilis, I have always regarded the cause as owing to persistent subclinical infections.[8] In other words, the condition may be similar to the persistence of agglutinins for typhoid bacilli after recovery from typhoid fever, which is so frequently attributable to the carrier state incident to persistent infections of the biliary tract with special reference to the gall bladder. Furthermore and, as previously stated, progression of latent into tertiary syphilis, with special reference to cardiovascular and/or neurosyphilis, occurs more frequently in seroresistant cases, which also indicates that seroresistance is apparently caused by persistent foci of infection. On the other hand, however, seroresistance may be attributable to the persistent production of reagin after biologic cure similar to the persistent production of antibody to pneumococcus polysaccharide. At any rate, Cannefax and Johnwick[9] have recently reported that the re-treatment of seroresistant cases of human syphilis does not hasten the reduction of reagin, and that re-treatment after initial adequate therapy appears to be unnecessary, since a group of untreated controls showed a similar average spontaneous reduction in reagin. Similar conclusions were arrived at by the Clinical Cooperative Group in relation to arseno-bismuth therapy. In the past, however, I have always given follow-up courses of arseno-bismuth compounds to prevent progression, and I shall continue to give follow-up courses of penicillin until additional data are available. Certainly, this course is both safe and reasonable, especially since it appears that *T. pallidum* does not acquire resistance to penicillin, as originally observed by Kolmer and Rule[10] and as confirmed by Probey,[11] in experimental syphilis in rabbits. The fact that acquired resistance has not been reported so far in the treatment of human syphilis must also be considered.

Needless to say, a large number of different treatment schedules have been employed since penicillin was first used in the chemotherapy of syphilis 10 years ago. During the past two years, I have used PAM and Bicillin L-A as the compounds of choice, the schedules employed in the treatment of different clinical types being summarized in TABLE 3. It will be observed that the total dosages are somewhat higher than those commonly employed. This treatment is attributable to the fact that I am dedicated to the principle of preferring the administration of "too much" rather than incurring the risk of administering "too little," especially for the prevention of clinical and/or serologic relapses and seroresistance.

Urticaria owing to allergic sensitization occurs much less frequently than formerly, since procaine penicillin G has come into widespread use. In my experience, mild attacks may be ignored. In severe urticaria, however, a hypoallergic penicillin mixture may be employed, and the oral administration of an antihistaminic compound may also be used before and after each injection or, if necessary, after desensitization.[12]

As previously stated, I always include the oral administration of potassium iodide in the treatment of latent, late-acquired, and late-congenital syphilis.

TABLE 3

TREATMENT SCHEDULES EMPLOYED BY THE AUTHOR*

Types	Total dosage (units)	Administration
Primary and secondary	3.6 million	1.2 million first dose, followed by 600,000 every 2–3 days for 4 doses.
Latent (early and late)	9.0 million	600,000 every 3–4 days for 15 doses.
Late benign	12.0 million	600,000 every 3–4 days for 20 doses.
Cardiovascular	21.0 million	300,000 for the first and second doses followed by 600,000 every 3–4 days for a total of 36 doses.
Neuro asymptomatic and syph. meningitis	9.0 million	600,000 every 3–4 days for 15 doses.
Paresis and taboparesis	24.0 million	600,000 every 3–4 days for 40 doses.
Tabes dorsalis	18.0 million	600,000 every 3–4 days for 30 doses.
During pregnancy	6.0 million	600,000 every 3–4 days for 10 doses; preferably before the seventh month.
Congenital: early	1.6 million	100,000 every 3–4 days for 16 doses.
Congenital: late	12.0 million	300,000 every 3–4 days for 40 doses or 600,000 every 3–4 days for 20 doses.

* PAM or Bicillin L-A.

Some clinicians prefer to initiate the treatment of syphilitic aortitis with four intramuscular injections of bismuth salicylate administered at weekly intervals to prevent or reduce the severity of Jarisch-Herxheimer reactions. While these reactions are usually relatively mild and may not occur at all in uncomplicated aortitis, it is my custom to initiate treatment with two doses of 300,000 units each three or four days apart, especially in patients having paresis and taboparesis, both of which increase the chances of severe Jarisch-Herxheimer reactions. The danger of therapeutic parodoxical reactions, which are usually impossible of differentiation from progressive cardiovascular syphilis, also appears to be relatively insignificant and overemphasized. But since a total dosage of at least 20 to 25 million units may be required for the ultimate regression and healing of uncomplicated aortitis, I routinely administer 21 million units, with, however, little expectation of securing reversed serologic reactions.

Undoubtedly, the Dattner-Thomas concept of neurosyphilis is of great value in guiding and evaluating penicillin therapy, especially with reference to retreatment, on the basis of spinal fluid examinations. Certainly, every case of early syphilis should have at least one spinal fluid examination following treatment, and all cases of latent and late syphilis should be subjected to such an examination before treatment is given. In asymptomatic neurosyphilis and syphilitic meningitis, treatment with penicillin alone is sufficient. The same is true in tabes dorsalis although, in primary optic atrophy, it appears that treatment combined with penicillin and malaria is to be preferred. This combined treatment also appears to give somewhat better results than penicillin alone in the treatment of paresis and taboparesis, aside from the fact that initial treatment with malaria apparently reduces the incidence of severe and even disastrous Jarisch-Herxheimer reactions.

Needless to say, the administration of penicillin is quite safe during pregnancy, with little or no danger of producing abortions and miscarriages. Syphilitic mothers who have been adequately treated and are persistently seronegative need not be re-treated during subsequent pregnancies, although such treatment is advisable whenever there is doubt about the adequacy of previous treatment and in women who are unwilling to remain under close observation.

On the assumption that seroresistance may be owing to persistent infection, especially of the aorta and/or the central nervous system, it is my custom to give two courses of penicillin treatment per year over at least five years in succession. Each course consists of the administration of 600,000 units of PAM or Bicillin L-A, every three or four days for ten doses, totalling 6 million units, along with the oral administration of potassium iodide. This procedure is followed in the hope of preventing or delaying progression of the disease.

Undoubtedly both mapharsen and bismuth act synergistically with penicillin in the treatment of experimental syphilis in rabbits.[13, 14] Favorable results with this combination therapy have also been reported by various investigators in the treatment of early human syphilis.[15-18] Other investigators, however, have reported that the results have been no better than those observed with penicillin alone.[19-21] Since much larger doses of penicillin are now being employed in the treatment of syphilis than was formerly the case, it appears that differences between the incidence of clinical and/or serologic relapses and reinfections in early syphilis treated with penicillin alone and with penicillin, mapharsen, and bismuth are so negligible that combined treatment may be regarded as unnecessary.

Unfortunately, penicillin by oral administration cannot be employed in the prophylaxis and abortive treatment of human syphilis. In my laboratory, at least, the total dosage required in experimental syphilis of rabbits has been 200,000 units/kg. corresponding to about 14 million or more units per 70 kilograms of body weight.[22] In this connection, however, it appears that the intramuscular injection of 1.2 or 2.4 million units of PAM or Bicillin L-A may prove satisfactory.

Other antibiotics. Since aureomycin, terramycin, and chloromycetin (chloramphenicol) are also known to possess treponemicidal activity, they may be of value in the treatment of syphilis, especially in cases resistant or allergic to penicillin. Thus, various investigators have reported favorable results with *aureomycin* orally, intramuscularly, or in combination not only in the treatment of early but of late syphilis as well, including neurosyphilis.[23-29] By oral administration, it appears that 2.0 to 4.0 gm. may be given daily in divided doses for a total of 50 to 70 gms. By intramuscular injection, the usual dose has been 0.05 gm. every 12 hours. In my laboratory, single doses of 0.250 gm./kg. of *terramycin* (oxytetracyline), corresponding to about 18 gms. per 70 kilograms, have not been completely curative in the treatment of experimental syphilis of rabbits.[30] Favorable reports, however, have been made in the treatment of human syphilis by oral administration in total doses of about 30 to 60 gms. over periods of 7 to 15 days, sometimes combined with intramuscular injections of bismuth.[25, 27, 31-35] Dunlop and Robinson,[36] however, have recently reported that 0.5 gm. by intravenous injection, once a day for

10 days in succession, does not appear justified in the treatment of early syphilis in view of the difficulty of administration and the proved efficacy of other modes of treatment. *Chloromycetin* (chloramphenicol) has likewise been favorably reported upon in the treatment of syphilis, including neurosyphilis, by oral and/or intramuscular administration in total doses of about 24.0 to 36.0 gms. over periods of 8 to 15 days.[27, 37-39] It is still too soon, however, definitely to appraise the therapeutic activity of these compounds, with the result that penicillin still remains the antibiotic of choice in the treatment of the great majority of cases of syphilis.

Frambesia (Yaws), Bejel, and Pinta

As is well known, trivalent arsenical and bismuth compounds have also been found effective in the treatment of frambesia (yaws), bejel, and pinta. Indeed, our own Nichols first discovered the curative activity of salvarsan in experimental yaws of rabbits about 44 years ago.[3] There can be no doubt, however, that penicillin is the compound of choice in the treatment of these treponemal diseases, especially with intramuscular injections of PAM. Certainly, this has been found true in yaws with dosage schedules similar to those employed in the treatment of syphilis.[28, 40-46] In this disease, aureomycin, terramycin, and chloramphenicol have also been successfully employed in treatment, although it is too soon to appraise the results in definite terms.[47-49] Penicillin has also been found highly effective in the treatment of bejel[45] and of pinta. In the latter disease aureomycin, terramycin, and chloramphenicol have been found less effective.[45]

Borreliar Infections

While the therapeutic value of the trivalent organic arsenical and bismuth compounds is questionable in the treatment of relapsing fever, caused by infections with *Borrelia recurrentis*, *B. novyi*, and *B. duttonii*, there can be no doubt that penicillin has proved highly effective not only in the treatment of this disease but, likewise, in the treatment of ratbite fever caused by infection with *B. muris* or *Spirillum minus*. The dosage schedules employed have been similar to those used in the treatment of early syphilis. Small doses may clear the blood of the *Borrelia* of relapsing fever in experimental infections of the lower animals, but large doses, 200,000 to 400,000 units/kg., are required for biologic cure because of persistent infections of the brain. Aureomycin has also yielded promising therapeutic results, and so has terramycin, especially in the treatment of cases resistant to organic arsenical and other compounds.[50-52]

In fusospirochetal diseases attributable to infections with *B. vincentii* and *Bor. buccale*, in symbiosis with *Fusobacterium plauti-vincentii*, such as Vincent's angina, trench mouth, tropical or phagedemic ulcer, gangrenous balanitis, bronchopulmonary spirochetosis, *etc.*, penicillin by topical application and/or intramuscular injection has likewise proven more effective in treatment than the organic arsenical compounds and bismuth.[53, 54] Favorable therapeutic results have also been reported with aureomycin, terramycin, and chloramphenicol, although troches of chloramphenicol are too bitter for clinical use.[48, 53-57]

Leptospiral Infections

In infectious jaundice or Weil's disease attributable to infection with *Leptospira icterhemorrhagica* or *L. canicola*, in Japanese seven-day fever, and in other leptospiral diseases, treatment with the organic trivalent arsenical and bismuth compounds has not stood the test of time; and the sulfonamide compounds have proven entirely ineffective.[58] Some investigators have reported that penicillin possesses therapeutic activity in experimental infections and in Weil's disease if administered before the onset of jaundice, in reducing fever, the severity of symptoms, and the duration of the disease.[59-60] Other investigators, however, have reported unsatisfactory results.[58, 61] On the other hand, aureomycin may be more effective and the compound of choice in chemotherapy.[61] Otherwise, it is doubtful if antibiotics, including streptomycin, possess therapeutic activity in the leptospiroses, with the result that there is still an urgent need for the production of newer and more effective compounds.

Bibliography

1. Marquardt, M. 1949. Paul Ehrlich. Heinemann Medical Books. London; 1951. Schuman. New York.
2. Thomas, H. W. 1905. Some experiments in the treatment of trypanosomiasis. Brit. Med. J. **1:** 1140.
3. Ehrlich, P. & S. Hata. 1911. The Experimental Chemotherapy of Spirilloses. Rebinan. New York.
4. Mahoney, J. F., R. C. Arnold & A. Harris. 1943. Penicillin treatment of early syphilis. A preliminary report. Venereal Disease Inform. **24:** 355; 1944. J. Am. Med. Assoc. **126:** 63.
5. Kolmer, J A. 1950. Penicillin in the treatment of experimental syphilis of rabbits. V. The synergistic or additive activity of penicillin injected intramuscularly with sodium iodide administered orally and intramuscularly. Arch. Dermatol. and Syphilol. **61:** 49.
6. Kolmer, J. A. & A. M. Rule. 1953. Penicillin alone and in combination with mercury succinimide and potassium iodide in the treatment of acute syphilitic orchitis of rabbits. Am. J. Syphilis, Gonorrhea, Venereal Diseases. **37:** 77.
7. Barnett, C. E. *et al.* 1954. Effect of treatment in late latent syphilis. Arch. Dermatol. and Syphilol. **69:** 91.
8. Kolmer, J. A. 1938. Serologic reactions and immunity in relation to infection and treatment of syphilis. Am. J. Syphilis, Gonorrhea, Venereal Diseases. **22:** 426.
9. Cannefax, G. R. & E. B. Johnwick. 1954. The observation of serologic response following re-treatment for seroresistance and theoretical considerations on the meaning of seroresistance. Am. J. Syphilis, Gonorrhea, Venereal Diseases. **38:** 18.
10. Kolmer, J. A. & A. M. Rule. 1946. Acquired resistance of *Treponema pallidum* to penicillin. Proc. Soc. Exptl. Biol. Med. **63:** 240.
11. Probey, T. F. 1953. Attempt to produce a penicillin-resistant strain of *Treponema pallidum* in experimental syphilis. Am. J. Syphilis, Gonorrhea, Venereal Diseases. **37:** 369.
12. Alexander, L. J. 1953. Desensitization of the penicillin-sensitive patient. Arch. Dermatol. and Syphilol. **68:** 323.
13. Eagle, J. *et al.* 1946. The synergistic action of penicillin and mapharsen in the treatment of experimental syphilis. J. Venereal Disease Inform. **27:** 3.
14. Kolmer, J. A. & A. M. Rule. 1947. The synergistic or additive activity of penicillin, oxophenarsine hydrochloride and bismuth and potassium tartrate. Arch. Dermatol. and Syphilol. **56:** 179; 1948. *Ibid.* **57:** 965.
15. Pardo-Castello, V. & O. A. Pardo. 1950. Treatment of early syphilis with penicillin and bismuth subsalicylate. Am. J. Syphilis, Gonorrhea, Venereal Diseases. **34:** 431; 1952. *Ibid.* **36:** 342.
16. Danbolt, N. & T. Berdal. 1950. Penicillin-arsenoxide-bismuth treatment of early syphilis. Acta. Dermato-Venereol. **30:** 354.
17. Jones, E. H., Jr. 1951. Observations on 360 cases of primary syphilis (treated with

penicillin alone or with penicillin plus mapharsen and bismuth). Ohio State Med. J. **47:** 131.

18. Chargin, L. *et al.* 1951. Treatment of early syphilis with penicillin, mapharsen and bismuth (combined therapy). Arch. Dermatol. and Syphilol. **63:** 104.
19. Alexander, L. J. *et al.* 1950. The treatment of primary and secondary syphilis with 4 new schedules. Am. J. Syphilis, Gonorrhea, Venereal Diseases. **34:** 420.
20. Johnwick, E. B. 1950. The treatment of neurosyphilis: penicillin alone versus penicillin plus arsenic and bismuth. J. Venereal Disease Inform. **31:** 303.
21. O'Brien, J. F. *et al.* 1952. Therapy of early syphilis with artificial fever in combination with mapharsen, bismuth, and penicillin. Am. J. Syphilis, Gonorrhea, Venereal Diseases. **36:** 451.
22. Kolmer, J. A. & A. M. Rule. 1954. Penicillin by oral administration in the prophylaxis and abortive treatment of experimental syphilis of rabbits. Am. J. Syphilis, Gonorrhea, Venereal Diseases. **38:** 176.
23. Irgang, S. & E. R. Alexander. 1948. Treatment of early syphilis with aureomycin. Bull. Harlem Hosp. **1:** 91; 1951. *Ibid.* **4:** 58.
24. Kierland, R. R. & P. A. O'Leary. 1951. Oral treatment of neurosyphilis with aureomycin. Progress report. Am. J. Syphilis, Gonorrhea, Venereal Diseases. **35:** 544.
25. Robinson, H. M. & H. M. Robinson, Jr. 1951. Terramycin in the treatment of syphilis. Am. J. Syphilis Neurol. **35:** 479; 1953. *Ibid.* **37:** 243.
26. Rodriquez, J. *et al.* 1949. Aureomycin and its effect in early stages of syphilis. J. Am. Med. Assoc. **141:** 771; 1951. Arch. Dermatol. and Syphilol. **63:** 433; 1952. *Ibid.* **66:** 59.
27. Taggart, S. R. *et al.* 1952. Treatment of syphilis with aureomycin and chloromycetin. Am. J. Syphilis, Gonorrhea, Venereal Diseases. **36:** 174.
28. Wilcox, R. R. 1949. Aureomycin: new treatment for syphilis? 1949. Brit. Med. J. **2:** 1076; 1951. Practitioner. **167:** 636.
29. Chen, C. H. *et al.* 1950. Ambulatory treatment of syphilis with aureomycin. J. Med. Assoc. Georgia. **39:** 237.
30. Kolmer, J. A. 1952. Terramycin hydrochloride alone and with mapharsen and bismuth in treatment of experimental syphilis of rabbits. Proc. Soc. Exptl. Biol. Med. **79:** 143.
31. Irgang, S. & E. R. Alexander. 1953. Treatment of early syphilis with terramycin. Am. J. Syphilis, Gonorrhea, Venereal Diseases. **37:** 247.
32. Robinson, H. M. & H. M. Robinson, Jr. 1951. Terramycin in the treatment of syphilis. Am. J. Syphilis, Gonorrhea, Venereal Diseases. **35:** 479.
33. Hendricks, F. D. *et al.* 1950. Terramycin in the treatment of venereal diseases. J. Am. Med. Assoc. **143:** 4.
34. Schoch, A. G. & L. J. Alexander. 1950. Terramycin in the control of venereal diseases. Ann. N. Y. Acad. Sci. **53:** 459.
35. Levaditi, C. & A. Vaisman. 1951. La terramycine nouvelle médication antosyphilitique. Presse méd. **59:** 849.
36. Dunlop, E. M. C. & R. C. V. Robinson. 1954. Intravenous terramycin in the treatment of early syphilis and granuloma inguinale. Am. J. Syphilis, Gonorrhea, Venereal Diseases. **38:** 24.
37. Olansky, S. *et al.* 1953. Intramuscular chloromycetin (chloramphenicol) in the treatment of venereal disease. Am. J. Syphilis, Gonorrhea, Venereal Diseases. **37:** 253; 1951. *Ibid.* **35:** 234.
38. Willcox, R. R. 1950. Chloramphenicol and the venereal diseases. Brit. Med. J **1:** 467.
39. Whitehill, R. & R. Austrian. 1944. The treatment of primary and secondary yaws with penicillin. Bull. Johns Hopkins Hosp. **75:** 232.
40. Robinson, H. M. & H. M. Robinson, Jr. 1949. Studies on chloramphenicol in early syphilis. Southern Med. J. **42:** 988; 1949. Am. J. Syphilis, Gonorrhea, Venereal Diseases. **33:** 509.
41. Divindelle, J. H. *et al.* 1946 Preliminary report on the evaluation of penicillin in the treatment of yaws. Am. J. Trop. Med. **26:** 311.
42. Hill, K. R. *et al.* 1946. Treatment of yaws with penicillin. Lancet. **2:** 522.
43. Arje, S. L. 1947. Yaws treatment with single massive doses of penicillin. U. S. Naval Med. Bull. **47:** 965.
44. Guimaraes, F. N. 1951. Procaine penicillin in early yaws. J. Investigative Dermatol. **16:** 77.
45. Rein, C. R. & D. K. Kitchen. 1953. Mass eradication treatment of treponemal diseases with penicillin. Laboratory and clinical basis for selection of effective sched-

ules. Am. J. Syphilis, Gonorrhea, Venereal Diseases. **37:** 37; 1950. J. Investigative Dermatol. **14:** 239; 1952. *Ibid.* **18:** 137.
46. Hill, K. R. *et al.* 1951. Atlas of framboesia. Bull World Health Organ. **4:** 201.
47. Schaeffer, K. *et al.* 1951. The effect of orally administered aureomycin on *Treponema pertenue* in man. Am. J. Trop. Med. **31:** 24.
48. Loughlin, E. H. & A. A. Joseph. 1951. Terramycin in the treatment of yaws and tropical ulcer. Antib. and Chemotherap. **1:** 76.
49. Payne, E. H. *et al.* 1951. Chloromycetin as a treatment for yaws and tropical ulcer. Antib. and Chemotherap. **1:** 88.
50. Gilchrist, W. S. 1950. Aureomycin and relapsing fever. Lancet. **1:** 515.
51. Yeo, R. M. 1950. Tick-borne relapsing fever: its treatment with aureomycin. S. African Med. J. **24:** 457.
52. Gefel, A. & R. Rubenow. 1953. Terramycin in treatment of relapsing fever. Jerusalem-Tel Aviv. **44:** 263.
53. Hamm, W. G. & G. Ouary. 1944. Penicillin therapy in phagedenic ulcer. U. S. Naval Med. Bull. **43:** 981.
54. Gutch, C. F. 1947. Local penicillin therapy for tropical ulcer. U. S. Naval Med. Bull. **47:** 801.
55. Ampofo, O. & G. M. Findlay. 1950. Aureomycin in the treatment of yaws and tropical ulcer in Africa. Nature. **165:** 398.
56. Cipes, L. R. 1950. Aureomycin. Report of clinical use in 90 cases of bacterial infection in the oral cavity. N. Y. State Dental J. **16:** 450.
57. Caldwell, E. R. *et al.* 1950. The treatment of various infections with terramycin. J. Lab. Clin. Med. **36:** 747.
58. Hall, H. E. *et al.* 1951. Evaluation of antibiotic therapy in human leptospirosis Ann. Internal Med. **35:** 981.
59. Hart, V. L. 1944. A case of Weil's disease treated with penicillin. Brit. Med. J. **2:** 720.
60. Patterson, H. M. 1947. Weil's disease: observations in sixty-one cases with special reference to the use of penicillin in six cases. J. Am. Med. Assoc. **134:** 1077.
61. Batchelor, T. M. & G. M. Todd. 1950. Aureomycin and penicillin therapy in leptospirosis (Weil's disease). J. Am. Med. Assoc. **143:** 21.

CHEMOTHERAPY OF BACTERIAL INFECTIONS

By Robert J. Schnitzer
Chemotherapy Laboratories, Hoffman-La Roche, Inc. Nutley, N. J.

It may appear unjustifiable to include the subject of the chemotherapy of bacterial infections in this commemoration of Paul Ehrlich's scientific work, since Ehrlich never devoted himself to any large extent to this field of endeavor.[1] It is difficult to explain why, with a few exceptions such as studies on antibacterial activity of arsphenamine or the drug resistance of *V. cholerae* toward different dye stuffs *in vitro*, the published work of Ehrlich and his co-workers does not contain any indication that the master had taken a more than occasional interest in a field which has undergone such spectacular development both theoretically and practically during the past 40 years. I do not believe that the reason was merely the amount of work which was put into the development of trypanocidal and antisyphilitic drugs, nor should it be assumed that Ehrlich did not have available chemical compounds of antibacterial properties. As a matter of fact, he possessed and worked with quite a few of these compounds. The reason might more probably be found in Ehrlich's long-standing interest in immunological problems, as well as in the fact that his institute was officially the center for evaluating antitoxic and antibacterial immune sera. The consistent effect of immune sera in bacterial infections, as demonstrated in the serum-testing department, might have contributed to the impression that the approach to therapy of bacterial infections was primarily an immunological one. The early disappointments of other investigators, among them Emil von Behring, who tried phenolic disinfectants in experimentally produced bacterial diseases, was also, perhaps, not without influence on Ehrlich's outlook as to the possibility of the chemical cure of diseases of bacterial origin.

The theoretical and methodological principles, however, which were developed in Ehrlich's therapeutic studies in protozoan and spirochetal infections and the fundamental discovery that chemical cure by antimicrobial compounds was at all possible, was sufficient to stimulate research on the field of the bacterial infections. It was certainly not accidental that two of Ehrlich's co-workers, Julius Morgenroth and C. H. Browning, first developed drugs of practical usefulness in bacterial infections.

In 1911, Morgenroth and Levy[2] found the first compound which possessed the property of controlling experimental infections of laboratory animals with pneumococci: optochin, or ethylhydrocupreine. It is perhaps permissible to use this opportunity to tell the story of this discovery. Morgenroth was interested at that time in the antimalarial activity of quinine and was looking for experimental methods as well as for derivatives of quinine having better activity. Testing methods for antimalarials, *e.g.*, the different forms of avian malaria, did not exist at that time, although some of the parasites were known. Fortunately, Morgenroth had in his laboratory a strain of *Trypanosoma brucei* which possessed a certain not very marked sensitivity toward quinine in mice and, in carrying out these experiments, he found that homologues of hydroquinine (= methylhydrocupreine) were of more marked activity.

In another corner of Morgenroth's small laboratory in the Charité Hospital in Berlin, in which diagnostic work was also performed, one of Morgenroth's assistants studied the bile solubility of pneumococci, involving quantitative techniques in which, instead of oxbile, which is not uniform, well-defined solutions of sodium taurocholate were used. When it was found accidentally that trypanosomes shared with pneumococci the property of dissolving in bile salts, Morgenroth undertook experiments in which quinine and quinine derivatives were used in experimental pneumococcal infections. Quinine itself was of only slight activity. The effect of methylhydrocupreine was more marked, but its next higher homologue, ethylhydrocupreine, was of very marked activity. Ethylhydrocupreine was the first compound which consistently protected mice, guinea pigs, and rabbits against fatal pneumococcal infections. This drug, which possesses all the disturbing toxicological qualities of quinine and, in higher doses, produces disagreeable symptoms of acoustical and optical side effects, has nevertheless been used all over the world for the treatment of pneumonia, pneumococcal meningitis, and corneal ulcus, with or without combination with antipneumococcal serum, until the discovery first of the sulfonamides and then of penicillin made it obsolete. But as recently as 1941, Tipson *et al.*[3] and Schmidt and Sesler[4] have published interesting studies on the antipneumococcal activity of derivatives of apocupreine. It is of historical interest that the first sulfonamide tested for chemotherapeutic activity was a derivative of hydrocupreine synthesized by Heidelberger and Jacobs in 1919, more than 10 years before Prontosil was found.

Shortly after the discovery of the first antibacterial agent, it was found in Ehrlich's laboratory that arsphenamine was a chemotherapeutic agent of appreciable value in experimental infections with *Erysipelothrix rhusiopathiae* (Bierbaum), and Schuster described its therapeutic effect in natural and experimental infections with *B. anthracis.*

It is of considerable interest that these first observations in the chemotherapy of bacterial infections were made in animal experiments. Wright found the *in-vitro* activity of ethylhydrocupreine after the results of the animal experiments were known and he also pointed out that this bacteriostatic effect was not inhibited by serum. The influence of serum on bacteriostatic systems has been recently revived and is used frequently for the evaluation of the antibacterial substances. One assumes that compounds, the *in vitro* bacteriostasis of which is not inhibited by serum, will also be active *in vivo* and *vice versa,* a supposition of doubtful validity.

Following Wright, Neufeld and Schiemann found that the chemotherapeutic activity of arsphenamine in swine erysipelas and anthrax could be correlated to a bacteriostatic *in vitro* effect of the drug on the causative organisms of these infections.

Wright's investigations, as well as Neufeld's, were of lasting influence on methodology in the field of the chemotherapy of bacterial infections. Since 1912, this technically simple, although probably biologically quite complex, method has been consistently used for the evaluation of antibacterial substances and even as a means of selection for compounds which might be further studied by animal experimentation. Whether this assumption is justified is another and somewhat controversial problem. While it is true that a majority of the known antibacterial drugs that possess the property of chemotherapeutic activity *in vivo* are also active *in vitro*, the reversal of this statement is not true. Morgenroth was the first to draw attention to this discrepancy which has subsequently been frequently confirmed, *e.g.*, by Marshall.[5] It is now well known that quantitative differences of *in vitro* bacteriostasis are not necessarily reflected by different potency *in vivo*.

Be that as it may, there is no doubt that the *in vitro* test has contributed considerably to the progress in chemotherapy of bacterial infections. The next step in this development was Browning and Gilmour's work on the antibacterial activity of acriflavine. This quaternary acridine dye stuff, which had been synthesized by Benda in Ehrlich's laboratory and had been thoroughly studied as to its interesting antitrypanosomal properties was found to possess remarkable antibacterial properties having what we should now call an unusually wide spectrum, involving gram-positive, but also, to a certain degree, gram-negative organisms. Shiga, in Ehrlich's laboratory, had shown that the dye stuff was active *in vitro* against *V. cholerae*. Browning's studies opened a new field of application of the chemotherapeutic principles in bacterial infections. This branch of chemotherapy, which Morgenroth called 'chemotherapeutic antisepsis,' was born of the requirements of surgery in the first World War and of the necessity of treating infected wounds or of protecting them against secondary infections. The disinfectants known at the time were soon found to be unsuitable for this purpose, perhaps with exception of Dakin's solution, but chemotherapeutic agents proved to be of considerable value. The agents were used not only for surface antisepsis, but also for the more or less successful attempts of sterilization of lesions deep in the tissues (Morgenroth's 'Tiefenantisepsis'), and were all characterized by marked *in vitro* bacteriostasis. Compounds having local efficacy in experimental tissue infections lacked almost completely chemotherapeutic activity in generalized infections, since they were without striking effect if applied systemically. To this group of compounds, which were studied experimentally and, in many cases, used clinically during the following 20 years, belong the higher homologues of Optochin, namely the isoamyl- and the isooctylhydrocupreine, both based on Morgenroth's work. The specific therapeutic properties of acriflavine stimulated studies on acridine dye stuffs which, as a rule, are characterized by marked antibacterial activity, though generally limited to gram-positive organisms. The large group of derivatives of 9-amino acridines, the investigation of which, by the way, led also to the discovery of the first acridine dye stuff having antimalarial activity, contained compounds of lower toxicity and less marked dye-stuff properties. Another group of compounds which were frequently studied was the series of triphenylmethane dye stuffs. Study of these compounds revived the early (1890) discovery made by Stilling of the local antibacterial effect of crystal violet or gentian violet. Neufeld and his co-workers have made a great number of investigations in this field. The generally high bacteriostatic effect of these dye stuffs is well known, and so is their toxicity. Whether or not their lack of effectiveness at systemic administration is entirely attributable to the fact that sufficiently high doses cannot be given is doubtful and will be discussed later. The antibacterial potency of these dye stuffs, particularly against staphylococci, has kept interest in them alive and, strange as it may seem, the local administration of gentian violet is still practiced.

As a rule, practical therapy using strongly colored and staining compounds has always met with resistance on the part of physicians and patients. In an attempt to replace the acridine dye stuffs by at least equally active but less highly colored or uncolored substances, the two investigators who seemed always most active in the field of chemotherapy of bacterial infections, C. H. Browning and J. Morgenroth, turned to another class of compounds: the quinoline derivatives. Browning and his co-workers, Cohen, Gulbransen, and others, studied a great number of quinoline derivatives, particularly quaternary styryl- and anilquinolines related to the group of cyanine dye stuffs, a type of dye stuffs widely used as photographic sensitizers. The new compounds possessed, therefore, more or less marked dye-stuff character, and some of them commanded considerable theoretical interest on account of their property as vital stains for parasite structures. Browning's work comprised an amazingly large number of compounds in which the influence of chemical variation on the activity was thoroughly investigated. Whereas the trypanocidal properties were in the foreground of interest in styrylquinoline compounds, high antibacterial activity was frequently encountered in the series of anilquinolines. One member of this group was 'Quinanil,' which exhibited antibacterial properties equal or superior to acriflavine. Its effect in generalized infections and under the conditions of systemic treatment was unsatisfactory. Related compounds, *e.g.*, the 6-aminocaproyl derivatives were described as exerting an effect in the streptococcal infection of mice if administered at the site of infection. This result seems to indicate that these compounds also had only a local effect in bacterial infections. The same thing was true for the numerous aminoquinoline derivatives studied by Morgenroth. Most of them did not even approach the activity range of acridine dye stuffs. After Morgenroth's early death, I continued studies on quinoline derivatives in the laboratories of the Hoechster Farbwerke in Germany which led to the finding of at least one compound of sufficiently high local antibacterial potency. This compound was a symmetrical urea of 4,6-diaminoquinaldine and belonged to the group of Jensch's[6] bisquinoline compounds which contained also other members of interesting chemotherapeutic qualities, particularly in trypanosomal infections.

If one tries to summarize the experience of this period of research, which has covered about 15 years, one might state that, during this time, probably many thousands of highly bacteriostatic agents have been studied, none of which has been found to possess the properties of a real chemotherapeutic agent in generalized bacterial infections. Their efficacy, both experimentally and clinically, was limited to local administration. The reason for the failure of these substances to influence generalized infections is not known. It is not only, as one has occasionally suspected, a matter of the toxicity of these compounds, many of which were quite atoxic; nor can the reason be insufficient absorption from the site of administration, for many of these compounds are highly active antitrypanosomal agents which readily, as a rule by a single injection, control trypanosomal infections or are able to influence intracellular infections caused by other protozoan parasites, such as babesia. Owing to the lack of comparative studies, no satisfactory answer to this problem can be given at present.

During the same period, two groups of chemotherapeutic agents were found which were able to influence acute infection from hemolytic streptococci by systemic administration. The *first* group consisted of organic *gold compounds* (1926). This discovery was surprising, since the early use of gold compounds (1890) suggested by Robert Koch, on account of the high *in vitro* activity of potassium cyanoaurate on *M. tuberculosis*, had not been convincingly successful, either in experimental infections in guinea pigs or in natural infections in humans. Also Moellgard's extensive studies on sodium aurothiosulfate or Sanocrysin did not prove that gold was an active antibacterial agent in tuberculous infections. It seemed that gold therapy of tuberculosis, at least in chronic productive cases, had some influence on the course of the disease, particularly by stimulating mesenchymal reactions, thus increasing the fibrotic processes which tended to obliterate the infected area, but a chemotherapeutic effect, in the sense of a direct influence on the causative mycobacteria has practically never been established. It was surprising when Schiemann and Feldt first described, in 1926, the phenomenon of consistent protection of mice fatally infected with streptococci by a single injection of a gold compound. The first compound to show this effect was Sanocrysin, but it has subsequently been confirmed that other acyclic and cyclic gold derivatives, all containing a goldthiol group, exert similar effects. Apparently these effects can also be observed in infections caused by *E. rhusiopathiae*, and in experimental infections attributable to salmonella and pasteurella in rabbits. It might also be mentioned that the activity of gold compounds comprises also the pleuropneumonia-like organisms, particularly infectious arthritis in rats, as was first shown by Collier[7] and later by Sabin and Warren.[8]

In vitro activity against bacteria is common in the case of inorganic gold compounds. Of the goldthiol derivatives of cyclic aromatic compounds, some are active *in vitro*, some are not; a correlation of *in-vitro* and *in-vivo* activity could rarely be established. The clinical usefulness of gold compounds was always difficult to evaluate on account of toxic side reactions. These compounds, however, have been found effective in treating certain forms of arthritis and are perhaps still employed in this condition.

The second group of compounds to show chemotherapeutic effects in experimental infections with hemolytic streptococci were acridine dye stuffs. As had been mentioned before, most of the acridines were local antiseptics which had no effect in systemic infections. Among many hundreds of acridines which I have investigated, only one group was found to possess chemotherapeutic properties in generalized infections. This group comprised a series of substituted 2-nitro-5-aminoacridines which were able to control acute streptococcal infections of mice if given parenterally or orally in high doses.[9] Although these substances were of comparatively low toxicity for laboratory animals, they had all the undesirable properties of acridine dye stuffs, such as staining, tissue irritation, and other toxic by-effects which interfered with the use of these compounds despite their effectiveness.

The historical development of the chemotherapy of bacterial infections, which I have attempted to present to you in this part of the paper, comprises the first 20 years of research. The efforts of investigators had resulted in demonstrating the fact that, in bacterial infections, chemical control of experimental and natural diseases was possible, similar in principle to the achievements in protozoan and spirochetal infections. Three groups of compounds had been found to exert reproducible chemotherapeutic effects, namely: the cupreines, having a marked specificity toward pneumococci; certain nitroacridines, having a specific activity on streptococci; and the metal compounds containing gold or arsenic, showing efficacy against streptococci and *E. rhusiopathia* or *B. anthracis* respectively. Although the effectiveness of all these compounds was less marked than that of the chemotherapeutic agents we know today, this early work had paved the way for them by establishing the fact that the same principles which Ehrlich had evolved in the chemotherapy of protozoan infections can be applied also to the diseases caused by bacteria. Evidence was accumulated that chemical agents, owing to a combined physicochemical and chemical relation to microorganisms, were able to interfere with the pathogenic qualities of the bacteria, to suppress their growth, and eventually to sterilize the blood and tissues of the host without interfering with its life and well-being. The characteristic property of the bacteria to develop drug resistance against all the agents known at that time was also well established.[10] The only things still missing were antibacterial agents of high activity and low toxicity. The spectacular development of the chemotherapy of bacterial infections during the following 20 years showed that such agents did exist. The biological properties of these compounds were so convincingly superior to those of the previously studied drugs that their general use met little of the usual resistance.

The right compounds had obviously been found at the right time, and they had opened the road to a new era of the chemotherapy of bacterial infections. The two groups of agents destined to establish antimicrobial therapy firmly as a means of controlling bacterial infections, namely the sulfonamides and the antibiotics, *i.e.*, the antibacterial substances of microbial origin, were discovered at about the same time. The fact that the sulfonamides, comparatively simple and generally readily accessible synthetic substances, were more easily available, made them the first successful drugs at a time when the first antibiotic, penicillin, was still in the stage of a rare hypothetical material of unique properties, but chemically undefined and lacking even a procedure of production.

The period of chemotherapy using sulfonamides started with Domagk's discovery of the antistreptococcal activity of a simple azo-dye stuff (4′-sulfamyl-2,4-diaminobenzene HCl) known by the trade name Prontosil. This substance, which was of low toxicity for laboratory animals and produced only slight by-effects, if any, in humans, was appreciably active in experimental infections of mice caused by hemolytic streptococci if given by the oral or parenteral route. The antipneumococcal effect, although less marked, was at least promising, and its efficacy was soon confirmed in the therapy of coccal infections in man.

The great progress which followed the initial discovery of Prontosil was based on the observation of Tréfouel, Nitti, and Bovet that the dye stuff molecule was not essential for the chemotherapeutic effect of the new drug, but that the simple sulfonamide moiety was the active principle. This compound, sulfanilamide, first synthesized by Gelmo in 1908, became, for the development of antibacterial chemotherapeutic substances, as important as arsanilic acid for the development of the chemotherapy of syphilis. Sulfanilamide possessed all the therapeutic properties first observed with its azo derivative. It produced, however, certain toxic side-effects, one of them sulfhemoglobinemia, which limited its prolonged use in some instances. This problem was a minor one, because the chemical configuration of sulfanilamide lent itself readily to chemical variation. From this work resulted the therapeutically valuable sulfonamides which are now in use.[11] All sulfonamides are fundamentally N′-substituted derivatives of sulfanilamide. The heterocyclic substituents as used in sulfapyridine (Whitby), sulfathiazole (Fosbinder and Walter), and, particularly, the pyrimidine derivatives synthesized by Roblin, Williams, and English and biologically first evaluated by White, namely sulfadiazine, sulfamerazine, and sulfamethazine, surpass the sulfanilamide by their lower toxicity and by a wide range of antibacterial activity. Other sulfonamides of similar constitution and of similar therapeutic qualities, such as Hoffer's sulfisoxazole (Gantrisin[12, 13]) were found valuable on account of the absence of certain toxicological properties. All these sulfonamides are characterized by the lack of the specificity which was a theoretically interesting but practically a not very desirable property of the earlier chemotherapeutic agents. The efficacy of the sulfonamides covers a wide range of microorganisms, particularly all pathogenic cocci, aerobic, and anaerobic spore formers, and gram-negative organisms of the coli-, shigella- and salmonella- group. There seems to exist a certain degree of specificity inasmuch as not all sulfonamides are equally active against infections a tributable to all different organisms, particularly in laboratory tests. It is also not claimed that all infectious diseases caused by these bacteria can be successful treated with sulfonamides. The salmonella infections, particularly those attributable to *E. typhosa* and most paratyphoid infections are still difficult to control in humans, although the causative organisms respond well to sulfonamides *in vitro* and in the more or less unnatural infections of laboratory animals.

The type of antibacterial effect of the sulfonamides is considered to be a bacteriostatic one, an opinion which seems to be based principally on observations of the *in vitro* test, where bactericidal activity seems to be absent. The apparent necessity of repeated administration of the drugs in animal experiments and the fact that surviving organisms were isolated after treatment with the earlier and less active sulfonamides seemed to suggest that the effect of these drugs *in vivo* is also bacteriostatic. Ercoli *et al.*[14] have shown that in the staphylococcal infection of mice the method of repeated treatment occasionally represents an unnecessary overdosage and that single treatment was markedly effective. The same thing is true of the effect of Gantrisin in staphylococcal and meningococcal infections and in experimental infections with members of the coli-salmonella group.[12] Whether this effect has something to do with the fact that all these infections were unnatural ones requiring the injection of gastric mucin in order to lower the host resistance awaits investigation. Also the question of lacking or incomplete sterilization of infected animals treated with sulfonamides seems not to be a firmly established fact. As in earlier studies with acridines, it seems not impossible that compounds showing bacteriostatic activity *in vitro* can be bactericidal *in vivo*, an observation which seems also to apply to penicillin (Grunberg *et al.*[15]). Grunberg and his colleagues found that about five hours after treatment of streptococci with penicillin *in vivo* the effect of the antibiotic could no longer be interrupted by hydroxylamine or penicillinase.

Returning to another aspect of the sulfonamides, one should mention two groups of compounds which show some essential differences from the drugs discussed so far.

The first group consists of compounds which are not antagonized by p-aminobenzoic acid. The first compound of this type was 4-(Aminomethyl)-benzene sulfonamide hydrochloride or Marfanil (Domagk), characterized by a marked activity towards anaerobic organisms. The lack of antagonism towards PABA, by the way, has not been confirmed by all investigators. Marfanil, however, is one of the few compounds in the sulfonamide group to show a marked local antiseptic effect *in vivo*. The other series of compounds were sulfanilanilids which, despite their high anticoccal *in-vitro* activity,[16] failed to show satisfactory activity *in vivo* owing to poor absorption and to the formation of an inactive conjugation product.[17]

The second group of sulfonamides to be discussed owes its value to the fact that its members are only insufficiently absorbed from the intestinal tract. Sulfaguanidine (Winnek) the first member of this group had been shown by Marshall, Jr., Bratton, White, and Litchfield to possess a marked effect on the bacterial flora of the intestines, although it is certainly absorbed and eliminated in the urine at least by certain individuals (Ingalls[18]). Sulfaguanidine was followed and more or less replaced by derivatives of sulfathiazole of similar properties such as sulfasuxidine (Welch, Mattis & Latven) and sulfathalidine (Mattis, Benson, & Koelle) drugs which became valuable as a means of reducing the bacterial flora for presurgical conditioning before operations involving the bowels. In acute intestinal infections, however, *e.g.*, in infections caused by *Shigella*, absorbed sulfonamides proved preferable.

According to Soo-Hoo *et al.*,[19] certain ketosulfones of p-aminobenzene sulfinic acid (Goldberg, Heinemann, and Grier[20]) exert an effect quite similar to that of the nonabsorbed sulfonamides. An interesting and theoretically important property of these latter compounds is their usefulness as a tool in nutritional experiments owing to the elimination of microbial vitamin production in the intestines.

Related to the sulfonamides in many respects are the sulfones, the parent substance of which, bis (p,p′-aminobenzene)sulfone (Buttle *et al.*), showed higher activity in coccal infections than sulfanilamide but was also, however, considerably more toxic. Its less toxic derivatives, such as the glucose derivative Promin and the rongalite derivative Diasone (Bauer and Rosenthal; Kolmer & Raiziss), were found to be active in experimental tuberculosis (Feldman *et al.*) and became the leading drugs in the treatment of leprosy.[21]

The discovery of the sulfonamides was of importance not only because these compounds were the first antibacterial substances of high therapeutic value, but also because it stimulated theoretically and practically interesting investigations on the fate of the compounds in the host organism, their distribution and elimination—problems which led in medical practice to a more rational use of the drugs. I am referring to Bratton and Marshall's method of determining sulfonamide concentrations in blood, serum, urine, and other body fluids and in the tissues. This technically simple and accurate method allowed a study of the relation of drug activity to the concentration of available drug in the blood and tissues, thus establishing a most valuable correlation for the determination of dose and effect on the basis of the actual drug distribution in the host (White). The practical significance of this contribution and its lasting influence on the elaboration of proper dosage schedules does not require further discussion. The experience with sulfonamides has caused continuous and successful efforts to establish similar tests for every new drug and has greatly assisted in the interpretation of the effect and the planning of the dosage of antibiotics.

Another development of far-reaching influence is connected with the chemotherapy of bacterial infections with sulfonamides: the studies of the mechanism of activity.[22, 23, 24] This field of research is based on Wood's finding that a metabolite of many microorganisms, p-aminobenzoic acid (PABA), antagonizes the antibaterial effect of sulfonamides *in vitro* and *in vivo.* From this discovery originates Fildes's theory that antibacterial activity is based on competitive interference with an essential bacterial metabolite of structural similarity. This theory, which has been widely confirmed and extended to many other fields, appeared to offer the first possibility to a rational approach in the field of chemotherapy (Woolley, McIlwain). The study of antagonists to metabolites, vitamins, and hormones, which I cannot possibly present in this review, has become a most valuable tool for the analysis of biological processes of growth and growth inhibition of micro-organisms. In 1950, an entire conference of this Academy was devoted to these problems.[25] The theory of Woods and Fildes was not uniformly accepted and interpreted by all investigators, but its fundamental meaning has not been invalidated in the 14 years since it has been published. It is, of course, true that the entire picture of the mechanism of sulfonamide activity is much more complicated (Martin lists about 40 known inhibitors) and that the PABA-antagonism involves a multitude of other aspects particularly in connection with folic acid, methionine (Kohn), respiratory enzymes (Sevag), transamination mechanism involving amino acids (Sevag and Steers), and nucleic acid derivatives of the pyrimidine series. It is impossible to go into details of this picture here, which in any case are extensively reviewed in good monographs.[22-24] Of the many theories which have been developed in addition to the original concept, however, one theory might be mentioned: that of Bell and Roblins,[24] which is an application of the ionization theory based on the PABA antagonism. This theory not only allows a calculation of activity on the basis of accurate measurements of dissociation constants, but also offers predictable activity rates at least for *Escherichia coli in vitro.* One had visualized that all these theoretically fascinating interpretations would lead the way into an era in which biochemical studies of bacterial metabolism would allow the planned chemical synthesis of chemotherapeutic agents of predictable activity. So far, the goal has not been reached, at least not in the field of bacterial infections. It seems that in other fields, namely, in the study of purines and pyrimidines (Hitchings[25]) it has resulted in more concrete developments, particularly as far as antimalarial and antimalignancy activity are concerned.

In case of bacterial infections, it seems still to be the rule that the observation of antibacterial activity precedes the elucidation of the mechanism of activity and that the knowledge of the latter, valuable as it may be, does not further considerably the synthesis of substances having predictable activity.

In the other and most recent chapter of antibacterial chemotherapeutics, the antibiotics, guiding principles based on theoretical considerations are more or less completely absent. The history of the discovery of penicillin by Fleming is as well known as the well-planned and tedious way in which Dubos developed the production of gramicidin and tyrothricin by using *B. brevis.* The first way seems to have been more or less accepted as a method for finding new active compounds. Since antibiotics are products of natural origin produced by penicillia, streptomycetes, and bacteria, the organisms producing antibiotics are isolated from soil or other natural products, and their antibacterial properties are studied and recognized by simple but ingenious methods of *in vitro* testing, by means of which the scope and range of their activity can be determined with satisfactory accuracy. It is apparent that antibiotic substances occur very frequently in nature and that most of those described so far and the range of their activities, which covers not only bacteria, but also fungi, protozoa, rickettsia, and viruses, indicate that unexpected discoveries of far-reaching importance can be expected by these studies. Of course, not all of the new antibiotics which are found are of practical value as drugs. As in the case of synthetic compounds, many different factors come into play, and it is generally a long time before an active compound found in the laboratory can be developed into a useful drug. Not all antibiotics that show activity *in vitro* are active *in vivo.* Many of these natural products, even in a state of high chemical purity, are extremely toxic. Large scale production frequently offers great problems. Nevertheless, more than a dozen antibiotics are practically used at the present time, compounds characterized by a wide range of chemotherapeutic properties and of considerable value in practical medicine.

The majority of these compounds are now chemically well-defined substances belonging to a great variety of chemical classes. The majority of them are large molecules, *e.g.*, Fleming's penicillin,[26] Waksman's streptomycin,[27] Duggar's aureomycin,[28] and the chemically related terramycin of Kane, Finlay, and Sobin.[29] The constitution of the polypeptide type antibiotics, such as the polymyxins (Benedict & Langlykke; Stansly *et al.*; Ainsworth *et al.*[30]) and Meleney's bacitracin[31] is not completely known. The only antibiotic of simpler structure is chloramphenicol (Ehrlich, Barth, Smith, Joslyn, and Burkholder,[32]) at the same time the only antibiotic which can be produced synthetically. Despite their different chemical structure, the antibiotics can show biological activity against the same bacteria or groups of bacteria, a characteristic attributable to the fact that the principle of specific activity which characterized the early antibacterial agents, already overcome by the sulfonamides, became practically nonexistent in most of the antibiotics. These compounds are representatives of anti-infectious agents of what is now called a broad spectrum, which not only covers a wide range of bacteria but also other infectious agents. Even if we limit ourselves to their antibacterial activity, the range of antibiotic efficacy is amazing. In FIGURE 1 are listed eight of the best known antibiotics and their *in vivo* activity against 16 bacteria or groups of bacteria. The statements as to the presence or absence of activity are based partly on our own experience and partly on published data from the literature. *In vivo* activity refers as a rule to laboratory findings, but in a few instances in which animal experiments have either not been undertaken or at least not been found in the literature, clinical effectiveness or failure have been used for the characterization. Since, however, the majority of our data were culled from experimental work, activity does not signify in all cases practical usefulness. An example of this inference is, for instance, the effect of penicillin on gram-negative organisms of the *Salmonella* group.

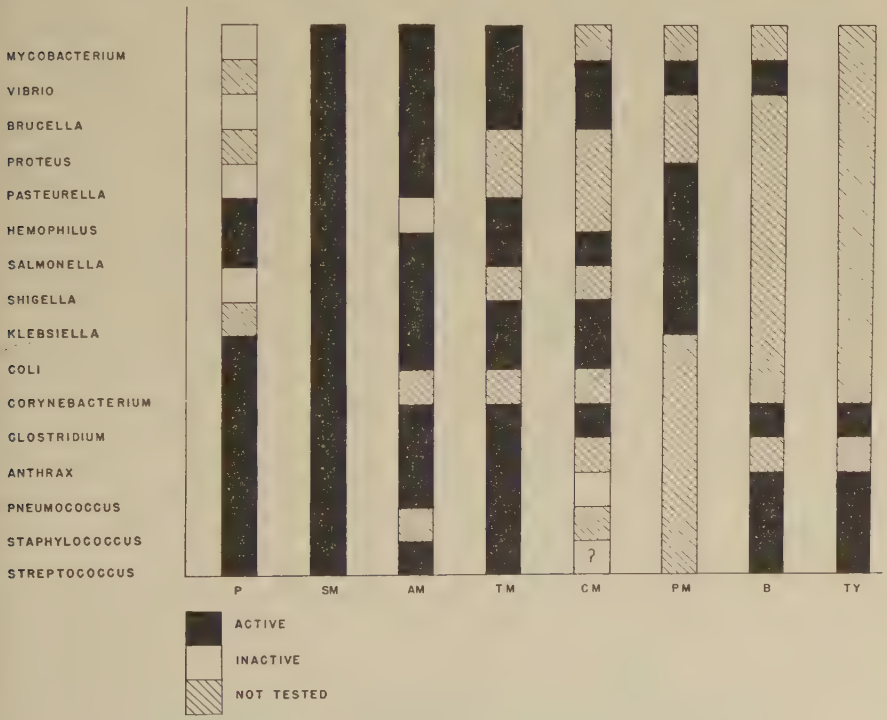


FIGURE 1. Antibacterial *in vivo* activity of eight antibiotics

P = Penicillin
SM = Streptomycin
AM = Aureomycin
TM = Terramycin
CM = Chloromycetin
PM = Polymyxin
B = Bacitracin
TY = Tyrothricin

A glance at the graph shows that, indeed, some of the antibiotics possess an extremely broad range of antibacterial activity, particularly streptomycin, aureomycin, and terramycin, to which group erythromycin, magnamycin, and related broad spectrum antibiotics have recently been added. Other antibiotics such as bacitracin or tyrothricin exhibit a marked specificity for gram-positive organisms, while chloramphenicol and the polymyxins influence to a higher degree infections with gram-negative organisms.

The graph does not indicate the quantitative relations of activity of the different antibiotics and it therefore does not allow differentiation in activity by degree. For the therapeutic use of antibiotics, this quantitative factor is, of course, of importance, although frequently only the *in vitro* sensitivity of the organisms, as measured by convenient plate assay methods, is used as a guide to rational therapy.

The mechanism of activity of the different antibiotics is not nearly so well known as that of the sulfonamides. Biological but occasionally also chemical reactions allow the determination of distribution and elimination, and are used for the evaluation of the antibiotics in blood, urine, and other body fluids. The problem of specific inhibitors which might indicate the type of inteference of the antibiotics with growth and viability of the sensitive organisms has not been elucidated in most instances. There is a considerable amount of literature to show the influence of cysteine on the *in vitro* effect of penicillin. The penicillinase production of certain bacteria and its influence on bacterial sensitivity has been studied, but there seems to be no specific theory at the present time to link the activity of penicillin to a specific metabolite or enzyme system. Most of this work was done *in vitro* with the exception of the interesting studies of Ercoli and his associates,[33] who showed that dimercaptopropanol (BAL), *i.e.*, a sulfhydryl compound like cysteine interfered with the activity of penicillin toward *Borrelia novyi* but not with the antibacterial *in vivo* effect toward streptococci and *Escherichia coli*. The same authors also confirmed the known antagonistic *in vitro* effect of cysteine on the bacteriostatic activity of chloromycetin by *in vivo* tests with BAL in the *Borrelia* infection. None of the *in vitro* antagonists of other antibiotics, *e.g.*, riboflavin antagonizing aureomycin or divalent cations antagonizing polymyxin have been shown to interfere with the therapeutic effect *in vivo*.

It is not my intention to belittle the value of these investigations, which may some day bring us nearer to an understanding of antibacterial activity and raise the tedious work of chemotherapeutic studies beyond the level of the entirely empirical screening which, unfortunately, is still the only promising way of finding, occasionally, an active substance which can be developed into a medicinally valuable drug. In order to illustrate this procedure, allow me to conclude this history of the chemotherapy of bacterial infections with a brief excursion in the field of recent developments in the chemotherapy of tuberculosis.

From the earliest attempts to influence experimental infections of laboratory animals with *M. tuberculosis* by chemotherapeutic treatment, which go back to the times of Robert Koch, to the discovery of the antituberculous activity of streptomycin by Waksman and his co-workers, the chemical control of this disease always appeared particularly difficult. One of the most important properties of the mycobacteria which seemed to cause their resistance to chemicals was the existence of a waxy outer layer of the bacterial organism. It had been assumed for a long time that chemotherapeutic activity of compounds would be dependent on the lipoid solubility of the substance and its ability to penetrate the waxy surface. It seems amazing that this theory could survive for such a long time since even *in vitro* tests indicated that many mycobacteria, particularly pathogenic forms such as *M. tuberculosis* responded to a great number of substances from different chemical classes. The recent very instructive monograph of Youmans,[34] in which the *in vitro* antitubercular activity of 3500 substances is described, shows clearly how frequent tuberculostatic activity *in vitro* occurs, and that there is no convincing correlation to lipoid solubility. It is true, of course, that this bacteriostatic effect could only be very rarely correlated to antituberculous activity *in vivo*. Many substances have never been tested *in vivo* but, in other cases, the lack of antituberculous *in vivo* effect of highly tuberculostatic substances seemed to demand another interpretation. This explanation was sought in the inaccessibility of the causative organism, and was based, at least partly, on the fact that the pathological substrate of the tuberculous infection is not vascularized. The tubercle bacilli in the necrotic center of a lesion walled off by the cells of the infectious granuloma could not be reached by the drugs. This concept was also not entirely correct. Already, in 1929, it had been shown by Jessen[35] that it was possible to stain vitally the necrotized center of tuberculous lesions of rabbits by intravenous injection of rosaniline. The ingenious studies of Florey's group[36] based on the direct observation of tuberculous tissue infections in the rabbit ear have convincingly shown that not only dissolved drugs but also insoluble compounds in the form of fine particles can reach the tubercle and be demonstrated in the necrotic tissue. These experiments were carried out with micrococcin, an insoluble antibiotic which possesses appreciable tuberculostatic activity *in vitro*, and although this material was present in the tuberculous lesion, it still failed to exert antitubercular activity *in vivo*. This situation makes it indeed difficult to characterize the properties which a remedy against tuberculosis should have in order to predict its activity.

Nevertheless, antitubercular *in vivo* activity is by no means a rare occurrence, and there are at least 13 compounds or groups of compounds known which possess this type of activity (TABLE 1). Before enumerating some of these substances I should like to point out that the efficacy of these compounds is based on experimental findings, particularly in the tuberculous infection of mice regardless of clinical usefulness.

At least five of these compounds are in clinical use, *e.g.*, p-aminosalicylic acid, pyrazinamide (aldinamide), viomycin, and, of course, streptomycin and isoniazid. Experience in the treatment of tuberculosis, particularly in chronic pulmonary forms of the disease, has shown that therapy with a single remedy is not always successful. One of the reasons is the development of drug resistance, which may interfere with the therapeutic effect of, particularly, the most active agents. It has been found advisable to use combined treatment with two or, if desired, three agents with different sites of antitubercular effect; a

TABLE 1[37]

Antituberculous compounds of low in-vivo *activity*

p,p′-diaminodiphenylsulfone (Feldman *et al.*)
p-aminosalicylic acid (Lehman)
nicotinamide (Chorine; McKenzie)
thioisonicotinamide (Gardner *et al.*)
pyrazinamide (Solotorowski *et al.*; Malone *et al.*)
4,4′-dialkyloxythiocarbanilides (R. J. Mayer *et al.*)

Antituberculous compounds of marked in-vivo *activity*

p-acetamidobenzaldehyde semicarbazone (Tibione) (Domagk *et al.*)
nicotinaldehydethiosemicarbazone (Levaditi; Grunberg)
Phenazines (V. C. Barry)
Phenoxazines (English; White)
Viomycin (Hobby, Lenert, *et al.*)

Antituberculous compounds of high in-vivo *activity*

Streptomycin (Waksman *et al.*)
Isoniazid (Grunberg & Schnitzer)

method which, by the way, had already been generally suggested by Ehrlich in 1907.[38]

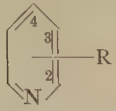
From a theoretical point of view, the existence of so great a number of appreciably active antitubercular substances makes it difficult to make any rational attempt of interpretation of the effect of these different compounds which (at least on the basis of drug resistance experiments) are representatives of agents having different mechanisms of activity.

It seems even less feasible to try to establish a correlation of chemical constitution and antituberculous activity which could be valid only in the narrow field of a few more or less closely related compounds. An interesting example in this respect are the derivatives of pyridine carboxylic acid. It had been shown in the three sections of the list that representatives of this class of compounds occurred in all three groups, from the low active ones to those of high activity. Grunberg has studied in the Roche laboratory isomers of four different groups of derivatives of pyridine carboxylic acids with somewhat surprising results. The data in TABLE 2 refer to results of experiments in mice infected intravenously with *M. tuberculosis* H37Rv and treated with the different therapeutic agents by diet. The figures represent the 50-per cent protective doses (PD_{50}) as evaluated in our standard experiment known from earlier publications[39, 40]

The table shows that antituberculous activity of generally moderate degrees has been observed in four out of the twelve compounds listed. In the series of amides, only the β-derivative was active; in the group of thioamides and hydrazines, the γ-derivatives. Thiosemicarbazones were found active as β- and γ-compounds, but in only one group, the hydrazines, some activity was observed with an α-substituted compound. I cannot see how the activity of any member of these quite closely related substances could have been predicted with any degree of probability. Moreover, there seems to be no indication except perhaps by the bacteriostasis *in vitro* which would have suggested the particular exaltation of efficacy which occurred in the isonicotinylhydrazine, isoniazid. Since the discovery of this drug, numerous and valuable contribu-

TABLE 2

ANTITUBERCULAR AGENTS OF THE PYRIDINE GROUP; INTRAVENOUS INFECTION OF MICE WITH H37RV



Activity (PD_{50}:mg/kg/day) *In Vivo* of

Position	$-CONH_2$	$-CSNH_2$	$-CH{=}N{-}NH{-}\overset{S}{\overset{\|}{C}}{-}NH_2$	$-CONHNH_2$
2	inactive	inactive	inactive	40
3	885	inactive	36	inactive
4	inactive	195	36	4.6

tions have been published in which the mechanism of activity has been studied. Investigations such as those of Mackaness;[41] of Suter,[42] who showed the surprising activity of isoniazid on intracellular tubercle bacilli, and of Barclay *et al.*,[43] who demonstrated the distribution of isotope-labeled isoniazid and the presence of the compound in or on the bacilli, have greatly contributed to our knowledge of the mechanism of activity. The fundamental problem, which is the most urgent question for everybody involved in the field of chemotherapy, namely the relation of chemical constitution and activity, has not even been brought near a solution. The complexity of the biological processes which come into play during the chemotherapeutic cure of an infection makes it appear very difficult to approach the problem experimentally.

This statement means that, even now, more than 50 years after the first demonstration of chemical cure in Paul Ehrlich's laboratory and, notwithstanding the progress of the chemotherapy of bacterial infections in the last 20 years, our knowledge of the fundamentals of our efforts is still very small; and honesty compels us to confess that, although we can find new drugs, we cannot create them.

References

Individual papers are generally given only if they are not contained in more extensive review articles, monographs, or handbooks.

1. MORGENROTH, J. 1914. Chemotherapeutische Studien. In Paul Ehrlich Festschrift. Fischer. Jena, Germany.
2. MORGENROTH, J. & R. LEVY. 1911. Berlin. klin. Wochschr. **48**: 1560; For older literature to 1934 see V. FISCHL & H. SCHLOSSBERGER. 1934. Handbuch der Chemotherapie. Fischer. Leipzig, Germany. R. SCHNITZER. 1926. Handbuch der biologischen Arbeitsmethoden. Abt. 8 (II) **2**: 115.
3. TIPSON, R. S., M. A. CLAPP & L. H. CRETCHER. 1943. J. Am. Chem. Soc. **65**: 1092.
4. SCHMIDT, L. H. & C. L. Sesler. 1941. J. Pharmacol Exptl. Therap. **72**: 311.
5. WHITE, H. J., A. C. BRATTON, C. T. LITCHFIELD & E. K. MARSHALL, JR. 1941. J. Pharmacol. Exptl. Therap. **72**: 112.
6. JENSCH, H. 1938. Z. angew. Chem. **50**: 891.
7. COLLIER, W. A. 1939. Z. Immunitätsforsch. **95**: 132.
8. SABIN, A. B. & J. WARREN. 1940. J. Bact. **40**: 823.
9. SCHNITZER, R. 1934. Medicine in its Chemical Aspects. **2**: 243.
10. SCHNITZER, R. 1932. Ergeb. Hyg. Bakt. Immunitätsforsch. Exptl. Therap. **13**: 227.

11. Northey, E. H. 1948. The Sulfonamides and Allied Compounds. Reinhold. New York.
12. Schnitzer, R. J., R. H. K. Foster, N. Ercoli, G. Soo-Hoo, C. N. Mangieri & M. D. Roe. 1946. J. Pharmacol. Exptl. Therap. **88**: 47.
13. Grunberg, E. & D. Eldridge. 1950. Yale J. Biol. and Med. **23**: 184.
14. Ercoli, N., M. N. Lewis & E. M. Harker. 1945. Am. J. Med. Sci. **209**: 621.
15. Grunberg, E., C. Unger & R. J. Schnitzer. 1948. Yale J. Biol. and Med. **20**: 479.
16. Schmidt, L. H. & C. L. Sesler. 1946. J. Pharmacol. Exptl. Therap. **87**: 313.
17. McChesney, E. W. 1947. J. Pharmacol. Exptl. Therap. **89**: 368.
18. Ingalls, J. R., Jr. 1954. Science. **119**: 191.
19. Soo-Hoo, G., N. Ercoli & R. J. Schnitzer. 1946. Jubilee Volume Barell. Basel, Switzerland. p. 358.
20. Goldberg, M. W., S. Heineman & N. Grier. 1946. Jubilee Volume Barell. Basel, Switzerland. p. 341.
21. Faget, G. H., R. C. Pogge, F. A. Johannsen, G. F. Dinan, B. M. Prejean & C. G Eggles. 1943. Pub. Health Repts. **58**: 1729.
22. Martin, G. J. 1951. Biological Antagonism. Blakiston. New York.
23. Sevag, M. G. 1946. Advances in Enzymol. **6**: 33.
24. Roblin, R. O. 1946. Chem. Revs. **38**: 255.
25. Woolley, D. W. 1950. Antimetabolites. Ann. N. Y. Acad. Sci. **52**: 8.
26. Florey, H. W., E. Chain, *et al.* 1949. Antibiotics. Oxford. London, England. New York. Toronto, Ont.
27. Waksman, S. A. 1949. Streptomycin. Williams & Wilkins. Baltimore, Md.
28. Duggar, B. M. *et al.* 1948. Aureomycin—A New Antibiotic. Ann. N. Y. Acad. Sci. 51(2).
29. Keefer, C. S. *et al.* 1950. Terramycin. Ann. N. Y. Acad. Sci. 53(2).
30. Long, P. H. *et al.* 1949. Antibiotics Derived from *Bacillus polymyxa.* Ann. N. Y. Acad. Sci. 51(5).
31. Johnson, B. A., H. Anker & F. L. Meleney. 1945. Science. **102**: 376.
32. Ehrlich, J., Q. R. Barth, R. M. Smith, D. A. Joslyn & P. R. Burkholder. 1947. Science. **106**: 417.
33. Ercoli, N., B. Gosford, G. M. Carminati, D. Kley & B. S. Schwartz. 1951. Proc. Soc. Exptl. Biol. Med. **78**: 253.
34. Youmans, G. P., L. Doub & A. S. Youmans. 1953. The Bacteriostatic Activity of 3500 Organic Compounds for *M. tuberculosis* var. hom. Chem. Biol. Coord. Center Natl. Research Council.
35. Jessen, P. 1929. Beitr. Klin. Tuberk. **72**: 108.
36. Florey, H. 1952. Proc. Roy. Soc. Med. **45**: 71.
37. Fox, H. H. 1953. Trans. N. Y. Acad. Sci. **15**: 234. H. H. Fox. 1953. Science. **118**: 497.
38. Ehrlich, P. 1909. Beitrage zur experimentellen Pathologie und Chemotherapie : 111–112. Leipzig, Germany.
39. Grunberg, E. & R. J. Schnitzer. 1952. Quart. Bull. Sea View Hosp. **13**: 3.
40. Grunberg, E. & R. J. Schnitzer. 1952. Yale J. Biol. and Med. **24**: 359.
41. Mackaness, G. B. & N. Smith. 1953. Am. Rev. Tuberc. **67**: 322.
42. Suter, E. 1952. Am. Rev. Tuberc. **65**: 775.
43. Barclay, W. R., R. H. Ebert & D. Koch-Weser. 1953. Am. Rev. Tuberc. **67**: 490.

DRUG RESISTANCE

By Harry Eagle
Section on Experimental Therapeutics, Laboratory of Infectious Diseases, National Microbiological Institute, National Institutes of Health, Bethesda, Md.

Of the many contributions to chemotherapy from Ehrlich's laboratory, one of the most important was the recognition of the phenomenon of drug resistance as a complication of chemotherapy, the fact that under certain conditions a drug could become ineffective because the microorganisms had become resistant to its cytotoxic action. It is therefore appropriate that on this occasion some of the current views on the basis of drug resistance be reviewed.* As will appear, the problem is far from being resolved; but it is nevertheless clear that the guiding principles laid down by Ehrlich are in large part still pertinent and valid.

1. *Adaptation vs. Mutation*

One of the most controversial aspects of the problem has been whether resistance is acquired by individual bacteria as a result of their exposure, or whether, instead, resistance arises in a relatively small number of organisms as a spontaneous mutation, the drug acting only as a selective agent which kills off the normal sensitive organisms, and thereby promotes the survival and selective propagation of the resistant mutant. The latter interpretation, that the increased resistance is a hereditary characteristic mediated by a modified gene, is consistent with classical genetic theory and general experience, and is strongly supported by evidence from a number of approaches. The studies of Demerec,[1] of Lederberg and Lederberg,[2] of Newcombe,[3] and of others have clearly established that organisms having a high degree of resistance to, *e.g.*, streptomycin are present in small numbers in the original population, without prior exposure to the drug.† Further, the demonstration by Hotchkiss[4] and by Alexander and Leidy,[5] that resistance to penicillin and streptomycin can be induced by exposure to desoxyribonucleic acid derived from resistant cells supports the thesis that an altered gene is responsible for the increased resistance. If resistance does in fact evolve solely as the result of spontaneous mutations, then the large quantitative differences in the rate at which bacteria become resistant to, *e.g.*, penicillin and streptomycin (FIGURES 1 and 2) reflect corresponding differences in the initial degree of heterogeneity in the population. That difference presumably reflects large differences in the degree of resistance produced by one-step mutations.

The possibility, however, has not been excluded that the minor degree of resistance often observed after exposure to threshold concentrations of drug

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* No attempt has been made to deal with all aspects of the problem under current study, or to cover the literature on any one aspect.

† Even the ingenious replicate plating technique of Lederberg does not exclude the possibility that the mutant differs from the normal sensitive cell, not in its *a priori* resistance, but in its capacity to adapt to normally lethal concentrations of the drug. There is no essential difference between these two explanations in relation to the question of the inheritance of acquired characters. However, they have quite different implications with respect to the biochemical mechanisms which may be involved in the phenomenon of resistance.

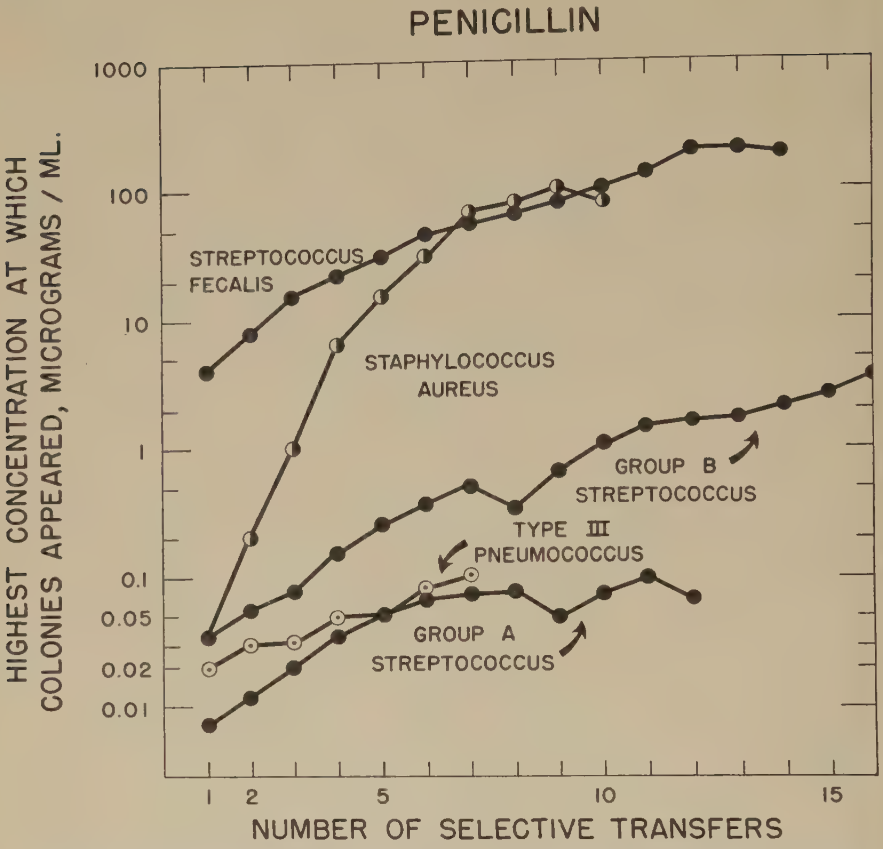

FIGURE 1. The development of increased resistance to penicillin by various bacterial species on repeated transfer through increasing concentrations of the antibiotic. In each passage, the organisms surviving in an agar medium at the highest concentration of antibiotic were subcultured and retested at appropriately higher concentrations.

may involve an adaptative response which affects a large proportion of the population. Implicit in this adaptation theory is the heretical consideration that an acquired character may be hereditary, for once resistance develops it may persist for many generations in the absence of the drug. One line of evidence that has been adduced in favor of the adaptation theory is the observation that the resistance of the organisms which grew out in the presence of drug is often related to the concentration of drug to which they had been exposed. At first sight, this would seem to imply a causal relationship. However, this is precisely what is to be expected from the distribution curves with respect to resistance (FIGURE 3). Even on the basis of selective multiplication rather than adaptation, a large proportion of the organisms which survive a low concentration would be sensitive to precisely the concentration to which they had been exposed and would be killed by slightly higher concentrations.

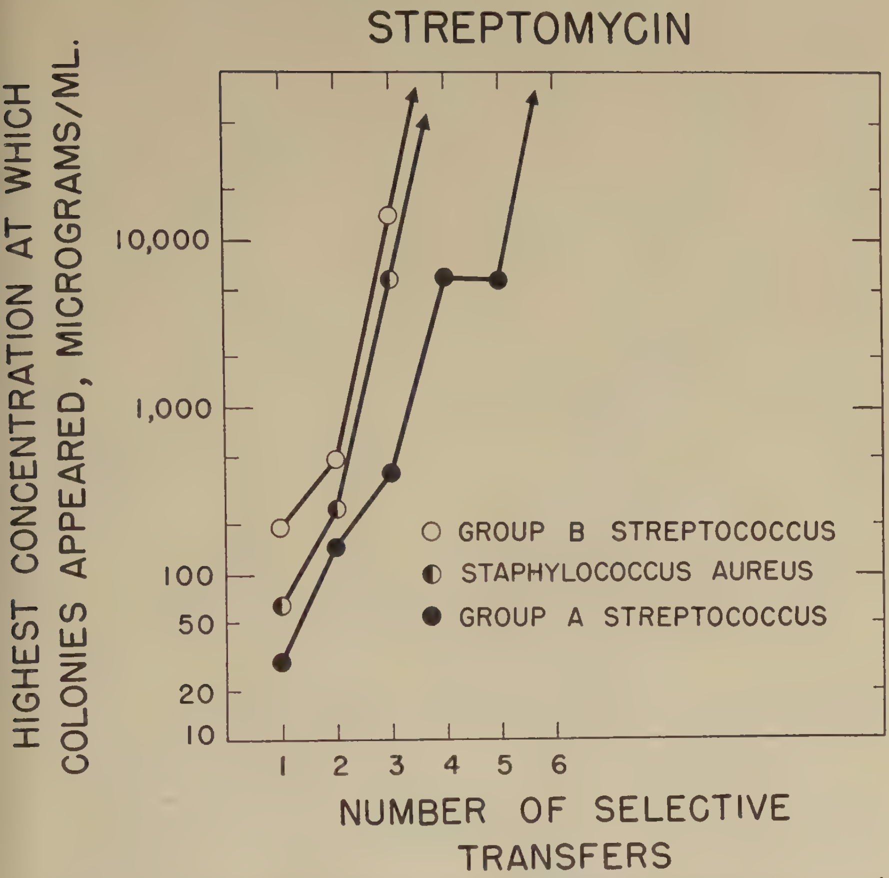

FIGURE 2. The development of increased resistance to streptomycin by various bacterial species on repeated tranfer through increasing concentrations of the antibiotic. In each passage, the organisms surviving in an agar medium at the highest concentration of antibiotic were subcultured and retested at appropriately higher concentrations.

In our laboratory, we have devoted considerable time and effort to this problem of mutation versus adaptation. . Although we have some suggestive evidence, we are not yet convinced that adaptation does in fact occur. In every instance in which a bacterial population has developed a minor degree of increase in resistance on exposure to threshold concentrations, there has been sufficient *interim* multiplication to permit the selective propagation of a relatively small number of resistant mutants. When a large proportion of a bacterial population is killed at a given threshold concentration of penicillin, simultaneous with the death of the sensitive organisms one can observe the progressive multiplication of the resistant variants (FIGURE 4). The sequence of events in this instance is clear. A somewhat more complicated situation is that illustrated in FIGURE 5, in which a bacterial population at threshold con-

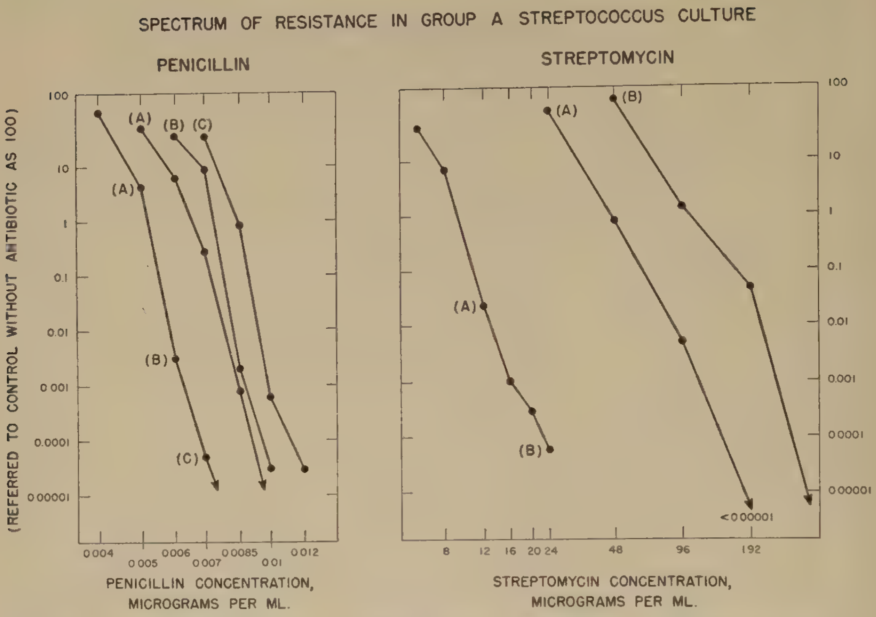

FIGURE 3. The spectrum of resistance to penicillin and streptomycin in a Group A streptococcus culture, and the increased resistance of the colonies growing out at various concentrations of the antibiotic. (Eagle *et al.*[18] reproduced by courtesy of the *Journal of Bacteriology*.)

centrations of chloramphenicol remained apparently unchanged in numbers over a period of days, but eventually began to multiply, coincident with the apparent development of resistance in the entire population. In this instance also, however, there had been a minor degree of multiplication, evidenced in the increasing number of organisms per clump, even though the plate counts remained essentially unchanged. It is entirely conceivable that, during this apparent lag period, resistant variants had appeared which then multiplied at a faster rate than the parent sensitive cells. The apparent transformation toward greater resistance would actually reflect the progressive replacement of the population by the resistant clone. Finally, when an attempt was made to "train" small numbers of staphylococci at threshold concentrations of penicillin, gradually increasing the amount of antibiotic in order to prevent multiplication, but slowly enough to avoid their death, one obtained the results illustrated in FIGURE 6. However small the increments of penicillin, the organisms either multiplied (too little penicillin) or died (too much penicillin). In no instance was there demonstrable adaptation to the drug. We have not yet observed the development of resistance in the absence of a degree of multiplication consistent with mutation and selection.

2. *Mechanisms of Resistance*

Let us now turn to a consideration of the difference between the sensitive and resistant cell which determines their varying susceptibility to a chemo-

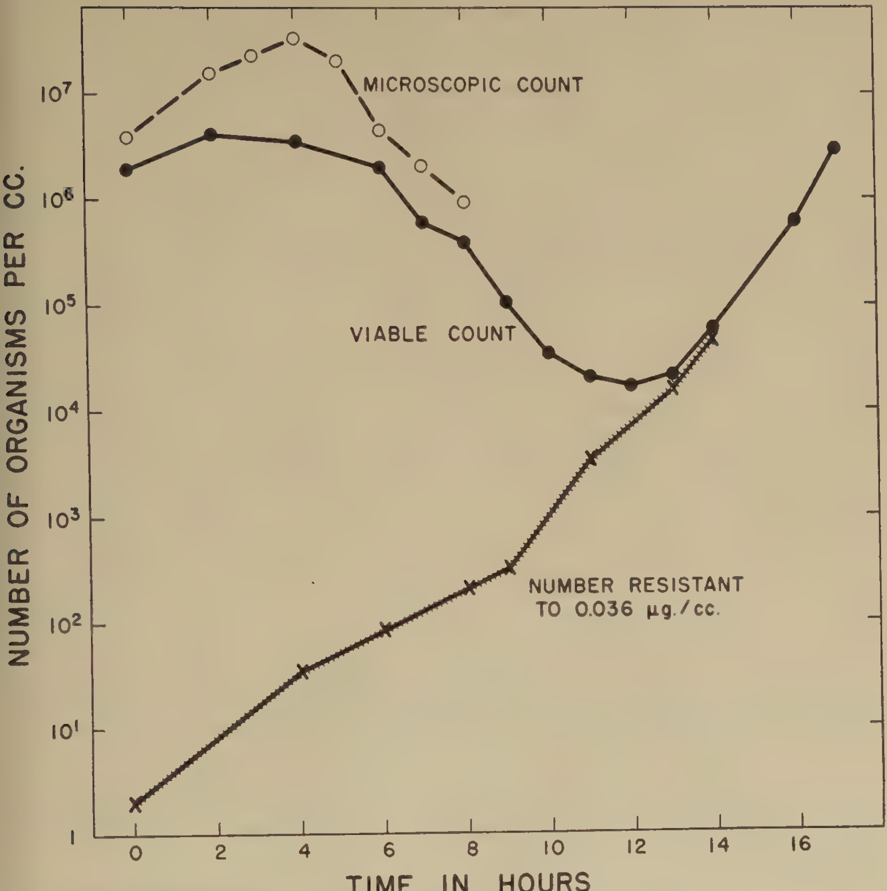

FIGURE 4. The development of resistance in a culture of *Micrococcus pyogenes* exposed to penicillin at 0.036 μg./cc. in a fluid medium.

therapeutic agent. One of Ehrlich's boldest concepts was the chemical approach to therapy exemplified by the thesis that "*corpora non agunt nisi fixata*," that chemotherapeutic agents do not exert their effect unless they have been bound by the specific chemoreceptors of the cell. For "chemoreceptors" we might today say enzymes or substrates or cofactors which mediate metabolic reactions vital to the cell, and which are functionally inactivated by their reaction with the drug. The corollary to the thesis that chemotherapeutic activity is determined by a reaction between the drug and a vulnerable cell constituent is that, in a series of homologous drugs, differences in cytotoxic activity may be related to differences in the degree to which the compounds are bound. This has received its most extensive test in the trivalent arsenicals and the correlation has, in fact, been highly satisfactory. In a large series of trivalent arsenicals, the degree to which the compounds

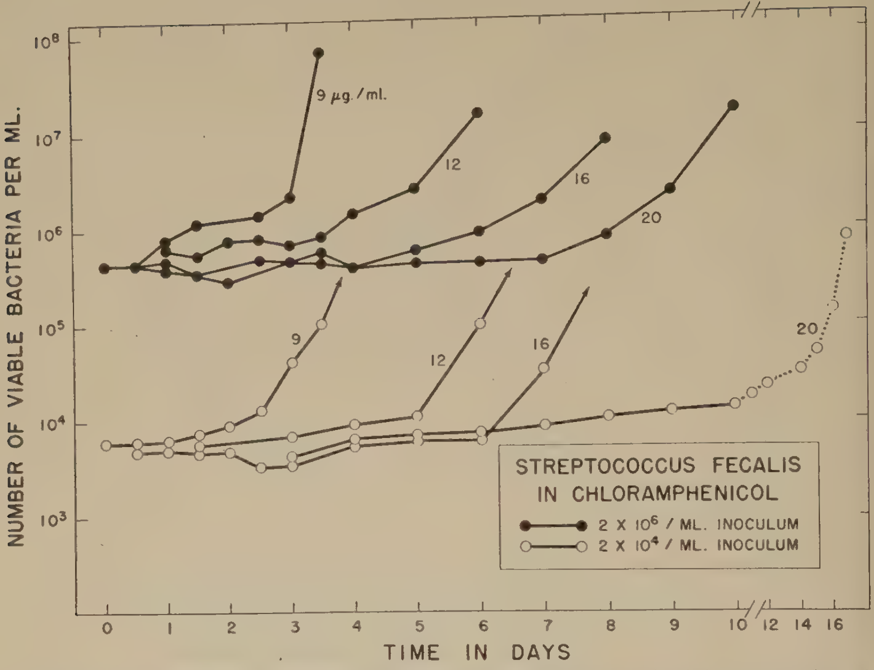

FIGURE 5. The development of resistance in *Streptococcus fecalis* exposed to threshold concentrations of chloramphenicol in a fluid medium. After a variable lag period during which the number of viable organisms, as determined by plate count, remained essentially unchanged, the organisms suddenly began to multiply, coincident with the apparent transformation of the total bacterial population to increased resistance.

were taken up by a suspension of trypanosomes varied directly with their activity against that strain.[6] Not only was this correlation between binding and activity valid with respect to parasiticidal action, but it extended to toxicity as well. The degree to which these trivalent arsenicals were taken up by a red blood cell suspension *in vitro* strikingly paralleled their systemic toxicity.[7] Presumably, the red blood cells served as a valid measure of the degree to which these compounds reacted with vital cell structures in the intact animal.

Ehrlich and his co-workers further showed that the development of increased resistance was often associated with the fact that the cell no longer bound the cytotoxic agent. Ehrlich[8] had found this to be the case with trypanosomes resistant to dyes; and shortly thereafter Levaditi[9] made a similar observation with arsenic-resistant trypanosomes. Yorke and his co-workers, in 1929–1931,[10] confirmed the fact that trypanosomes rendered resistant to arsenicals by selective transfer no longer combined with the drugs and, in our laboratory, we later showed this observation to be true with a spontaneously resistant mutant strain of *Trypanosoma equiperdum*.[11]

With penicillin also, the sensitivity of bacteria to the antibiotic is regularly associated with the ability to combine with the antibiotic. If suspensions of bacteria as they are isolated in nature (see below) are exposed to radioactive

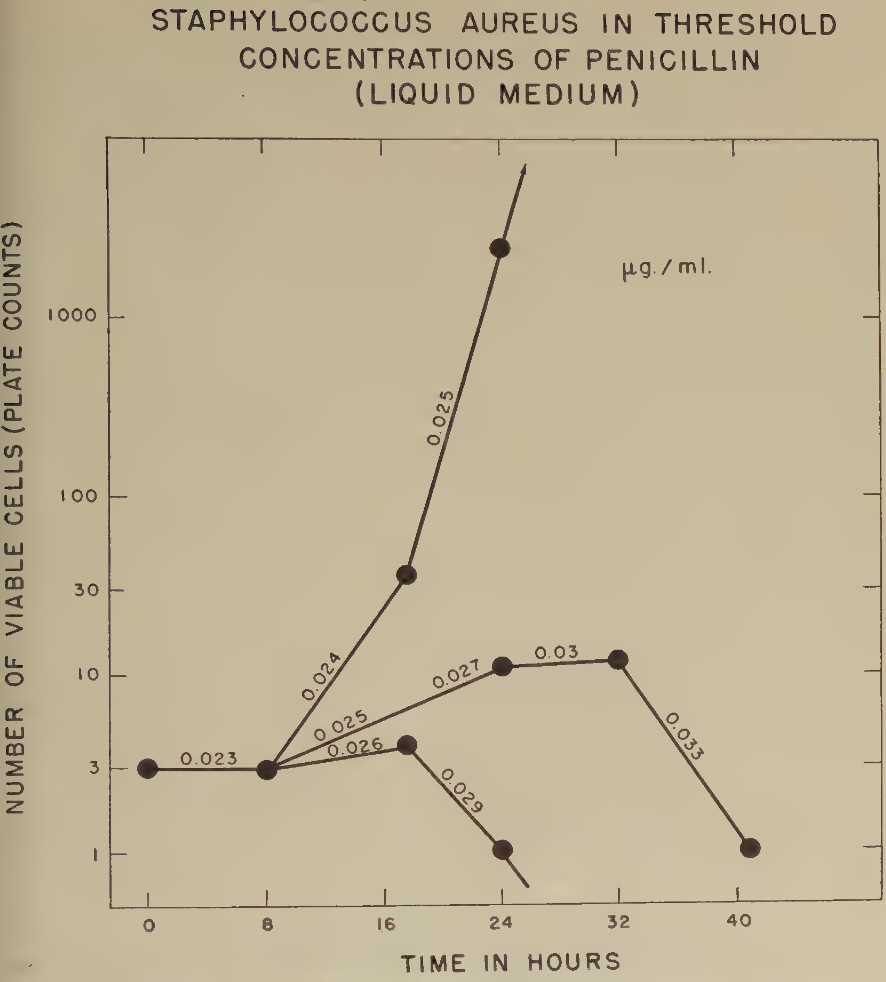

FIGURE 6. Illustrating an unsuccessful experiment to increase the penicillin resistance of *Micrococcus pyogenes* by slowly adapting the organisms to increasing concentration of the antibiotic.

penicillin under standardized conditions, the amount of antibiotic bound by the bacteria and retained after washing varies directly with their sensitivity to the drug[12] (FIGURE 7).

Why do die- or arsenic-resistant trypanosomes, and such relatively penicillin-resistant bacteria as *Escherichia coli*, *Staphylococcus fecalis*, combine with the chemotherapeutic agent to only a limited degree? The simplest explanation is that the resistant cells are relatively impermeable to the drug; and we had so interpreted our own results with the arsenicals. Recent results with penicillin suggest, however, that this may not regularly be the case. If cell-free extracts are prepared from naturally penicillin-sensitive and penicillin-

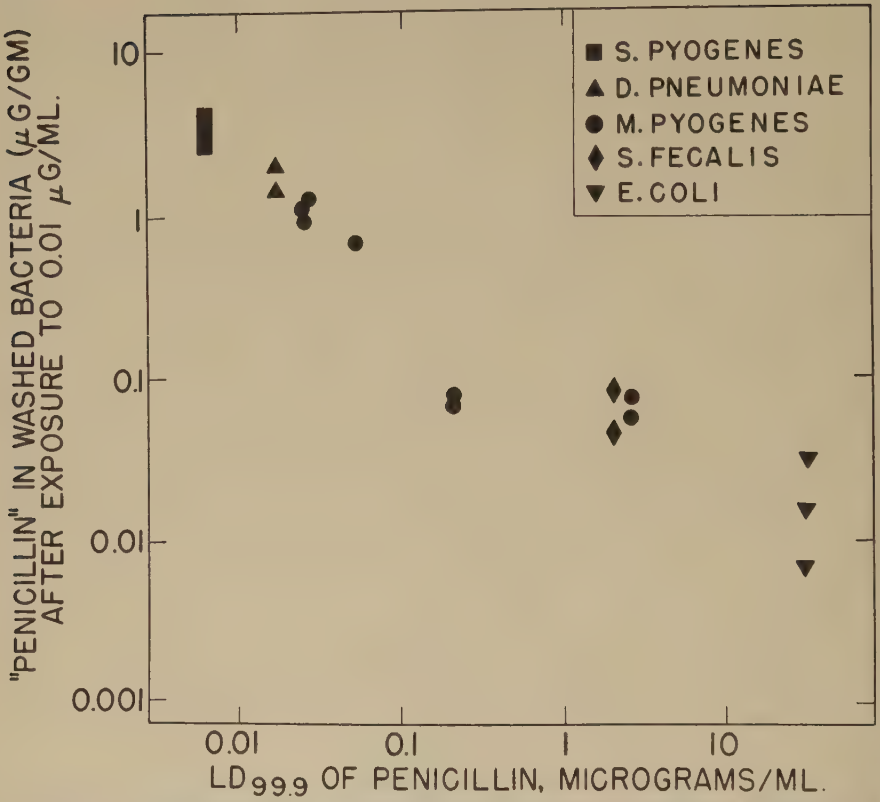

FIGURE 7. The relationship between the penicillin sensitivity of various bacterial species to penicillin and their combining affinity with the antibiotic. (Eagle[12] reproduced by courtesy of the *Journal of Experimental Medicine.*)

resistant bacteria, and if such extracts are incubated with radioactive penicillin, the components of the extract which were sedimentable in the ultracentrifuge, consisting largely of protein, bound penicillin to essentially the same degree as did the intact cells, and again in proportion to their sensitivity.[12] The high combining reactivity of penicillin-sensitive bacteria with the antibiotic and the relatively low reactivity of the penicillin-resistant organisms are therefore not attributable to differences in permeability.

Some of these relatively resistant bacterial strains have been found to degrade penicillin within the cell, even though they do not secrete penicillinase into the medium; and that inactivation, which is also observed in cell-free extracts, could explain both their resistance and the relatively small amounts of penicillin bound by the intact cells or by their extracts under standardized conditions. This reaction, however, is not a general explanation of resistance. With other strains which do not degrade penicillin, large differences in sensitivity are nevertheless observed which are correlated with the amounts of penicillin bound by the cells and by their cell-free extracts. With these organisms, changing sensitivity therefore appears to reflect differences in the actual reactivity of cell components with the antibiotic.

It is of particular interest that, no matter what the penicillin sensitivity of a given bacterial strain, the degree of combination necessary to effect death has proved quite uniform. For organisms having an $LD_{99.9}$ of less than 2 micrograms per cc., that lethal cellular concentration of penicillin varied only between 1.0 and 4.0 micrograms per gram dry weight, and from 1500 to 5000 molecules per cell.[12, 13] At concentrations higher than 2 micrograms per ml., there was uniform, nonspecific binding by sensitive and insensitive cells alike, which obscured the specific binding at lower concentrations.

Particular interest attaches to the reactivity of mammalian cells with penicillin. The nontoxicity of penicillin for the mammalian host is one of its most important and distinguishing characteristics. In keeping with the foregoing thesis, one would have anticipated that mammalian cells would not bind and markedly concentrate the antibiotic as do sensitive bacteria, but would instead react as do highly insensitive cells. This proved to be the case. As with insensitive bacteria, the intracellular concentration rarely exceeded that in the surrounding fluid. The failure of the mammalian cells to bind and concentrate the drug could not be related to their impermeability, for sonic extracts also proved to have an extremely low reactivity, and did not inactivate the antibiotic.[14]

The nature of the cell component or components which react with penicillin has also been under study. When *Micrococcus pyogenes* and *Staphylococcus pyogenes* were exposed to low concentrations of radiopenicillin (*e.g.*, 0.01 μg./ml.), and were then disintegrated in a sonic oscillator, from 85 to 90 per cent of the labelled penicillin taken up by the cell was found to be associated with water-soluble components which were not sedimented at 44,000 g. in 2 hours, and were presumably not large cell fragments. Of this, only 10 to 25 per cent was dialyzable. From 50 to 80 per cent of the bound penicillin was associated with large molecular weight components or particulates which were sedimented in the ultracentrifuge at 68,000 to 144,000 g. After tryptic digestion, a large proportion of this originally sedimentable material, varying in individual experiments between 40 and 90 per cent, could no longer be thrown down at these speeds.

The working hypothesis is suggested that the varying sensitivity to penicillin of bacterial strains and species as they occur in nature is often related to the reactivity of cell constituents with the antibiotic. Presumably, one or more of these is vital to the cell and is functionally inactiviated by that combination. The analogous components of the insensitive cell have a correspondingly low reactivity, and more penicillin is required in order to effect the lethal degree of combination.

It is entirely possible that, with trypanosomes also, the development of arsenic resistance may be related to a change in the reactivity of vulnerable cell components with the drug, rather than to a change in the permeability of the cell. Further, the varying activity of different arsenicals with the normal cell similarly may be related to differences in their chemical reactivity with cell components, and not to differences in the ease with which they penetrate into the cell. These aspects of arsenic resistance deserve reinvestigation.

The foregoing thesis, if correct, further illustrates the extraordinary prescience of Ehrlich who, 45 years ago, stated that "the chemoreceptors [of the cell] are related to the structure of its protoplasm, and are changed when that structure . . . is mutatively altered".[15]

The possible importance of the reactivity of vulnerable components of the cell with a cytotoxic agent as a determinant of sensitivity or resistance, however, must not be overemphasized. Even with the single agent penicillin, at least three other mechanisms are known to be involved in the phenomenon of drug resistance, and there may well be more. In the first place, it has been known for many years that certain cells can degrade penicillin by the production of an extracellular penicillinase, an enzyme which hydrolyzes penicillin to inactive penicilloic acid. Second, a number of bacterial strains which do not liberate penicillinase into the medium nevertheless have been found to inactivate penicillin within the cell. If the organisms are exposed to radiopenicillin, and if the sedimented bacteria are incubated prior to elution, the labeled material which is then extracted is biologically inactive. With some cell species, that intracellular inactivation proceeds at a sufficiently rapid rate to suggest that it may contribute to an important degree to the penicillin resistance of the cell.

Perhaps the most interesting aspect of penicillin resistance is the large segment of the problem which is not covered by any of the explanations already suggested. Omitting from consideration the bacteria which inactivate penicillin, the resistance of bacterial strains and species as they occur in nature is usually related to the reactivity of cell components with the antibiotic. However, if a strain which normally is highly sensitive to penicillin is rendered resistant by serial transfer through the antibiotic, then the resistant variants so produced often continue to bind penicillin to the same degree as the highly sensitive parent cell. Changes in binding capacity may and do occur, but independently of the development of resistance. In a few strains, binding capacity was decreased, as would have been expected on the basis of the foregoing discussion. With other strains, however, binding capacity was unchanged, and in some strains it was actually enhanced (FIGURE 8). With *Micrococcus pyogenes*, an initial increase in resistance was accompanied by increased binding activity, while a subsequent further increase in resistance was associated with a decreased binding activity. With *D. pneumoniae*, resistance was increased with no change in binding capacity. On continued passage in antibiotic, with no further change in resistance, the binding capacity of the resistant organism markedly decreased.[16] Some mechanism other than a decrease in the over-all reactivity of the cell with penicillin is clearly implicated. These resistant variants did not produce penicillinase, nor did they have an enhanced capacity to degrade the antibiotic within the cell.[16]

It is, perhaps, fruitless to speculate as to the mechanism of resistance in variants of normally sensitive cells. Conceivably, the cell component which is normally responsible for the death of the cell may, in the resistant cell, have a decreased reactivity with penicillin that is masked by the continued reactivity

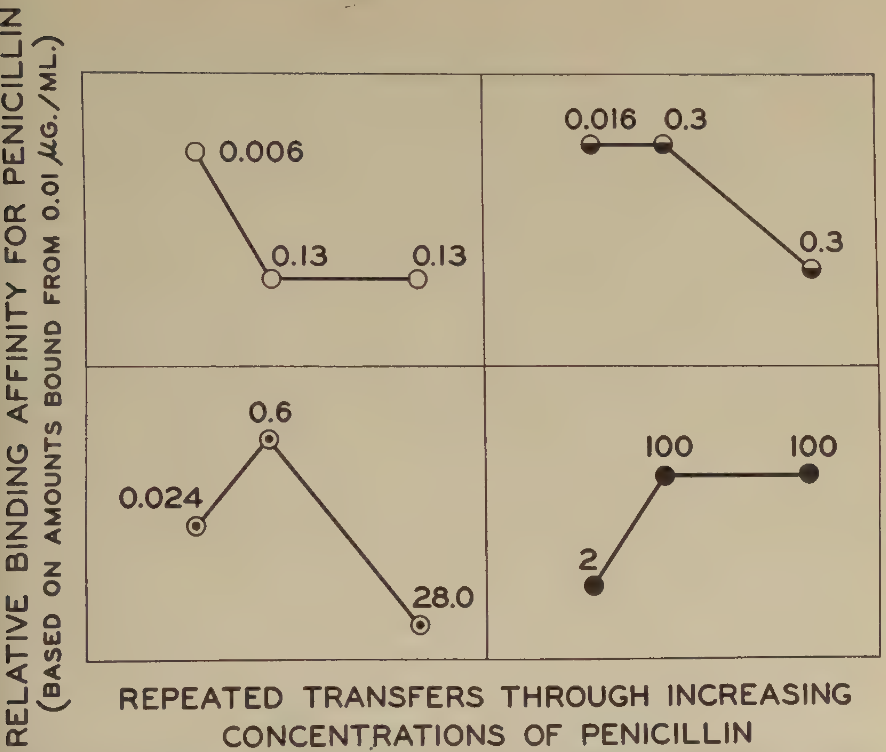

FIGURE 8. The absence of a regular correlation between the development of increased penicillin resistance (by selective transfer in increasing concentrations of antibiotic) and the binding affinity of the organisms for penicillin. (Eagle[16] reproduced by courtesy of the *Journal of Experimental Medicine.*)

of other components. The over-all combining affinity of the cell would then not be demonstrably altered. Alternatively, the resistant cell may no longer require the product of the metabolic reaction inhibited by penicillin, or may require it in reduced amount. A number of other possibilities will suggest themselves; nor is it certain that any of the mechanisms involved in penicillin resistance are operative with other agents.

There is considerable evidence that the antibacterial action of sulfanilamide usually rests on the fact that it competitively antagonizes a normal metabolite, PABA; and there is reason to believe that the development of resistance to sulfanilamide is often associated with an increased production of PABA. Studies on the binding of radioactive streptomycin and aureomycin by sensitive and resistant bacteria are now in progress.

3. *Clinical Considerations*

The complication introduced by the development of resistance in originally sensitive strains of infectious agents is self-evident. Thus, in some areas of the Belgian Congo, more than 50 per cent of the cases of trypanosomiasis no longer respond to treatment with tryparsamide. Similarly, the development of resistance to sulfanilamide by, for example, gonococci and streptococci, and the rapid emergence of streptomycin- and isoniazid-resistant strains of *Mycobacterium tuberculosis* have placed serious limitations on the therapeutic use of these agents. Since the introduction of penicillin, the proportion of resistant strains of staphylococci in hospital patients has progressively increased, to the degree that in some areas most of the strains now isolated are resistant to more than 1 microgram per cc. of penicillin.

Despite the importance of the phenomenon of drug resistance, its clinical impact as far as the antibiotics are concerned has perhaps been exaggerated. Certainly, the situation does not justify the dramatic descriptions in the lay press of the tendency of bacteria to develop increased resistance, and the discovery of new antibiotics just in time to fill the therapeutic gap so created. The problem presented by streptomycin and isoniazid has been largely if incompletely met by their use in combination with each other, or with p-aminosalicylic acid. For reasons which are not yet clear, the emergence of resistance is then greatly delayed. The therapeutic problem presented by antibiotic-resistant staphylococci is certainly a real one. It is, however, probable that this usually does not represent the emergence of resistance in originally sensitive strains, but rather the selective survival of strains that were resistant prior to the advent of the antibiotics, and which originally constituted only a small proportion of the normal human flora. Strains of staphylococci differ markedly in their resistance to penicillin. The wide-spread use of penicillin (not necessarily specifically in the treatment of staphylococcal infections) has apparently gradually killed off the relatively sensitive strains, so that penicillin-resistant strains now constitute a larger proportion of the organisms as they occur in a hospital population. It is significant that the resistant staphylococci now isolated from man are almost regularly producers of penicillinase, while the highly resistant organisms produced deliberately by selective transfer in increasing concentrations of penicillin neither produce penicillinase, nor have an increased capacity to degrade the antibiotic within the cell. There has been no clear demonstration that a strain of *Staphylococcus* initially sensitive to penicillin has, under the impact of therapy in a given case, become resistant.

With the exception of staphylococci, the development of increased resistance among organisms commonly treated with penicillin has been conspicuous by its absence. There has yet to be reported an authenticated instance of a penicillin-resistant group A Streptococcus, Pneumococcus, Gonococcus, or *Treponema pallidum*. Further, with at least some of these strains, and perhaps with all, it is unlikely that resistance will develop to the degree that penicillin will have lost its chemotherapeutic usefulness. In general, the development of resistance under treatment reflects an initial heterogeneity in the population. That heterogeneity may take either of two forms. Strains in nature may differ widely in their sensitivity to penicillin. As previously discussed, this is the case with staphylococci; but a similar degree of heterogeneity has not been found with any of the four genera enumerated above. The second form of heterogeneity represents differences in the sensitivity of individual bacteria of the same strain, either in a single culture, or in a single patient. With only a few exceptions, however, bacterial populations are remarkably uniform with respect to their penicillin resistance, as evidenced by the dosage-survival curve. With a group A *Streptococcus*, for example, 0.004 micrograms per cc. killed 20 to 80 per cent of the bacteria. A slight increase, to 0.008 micrograms per cc., killed more than 99.999 per cent; and the offspring of the survivors were killed by concentrations of 0.012 to 0.015 micrograms per ml. Such an organism is hardly likely to become resistant by the selective survival, in the patient, of the more resistant proportion of the population; for even the most resistant organisms in a population of 100,000,000 are only slightly more resistant than the average. In contrast, in the case of streptomycin, although 50 per cent of the same strain of group A streptococci were killed by a concentration of 6 micrograms per ml., even five times that concentration still permitted the survival of a few organisms; and subcultures of these survivors contained some organisms resistant to 100 micrograms per ml. Under these circumstances, the selection of the resistant variants by a single prolonged exposure to a concentration of, *e.g.*, 20 micrograms per cc. could result in a marked increase in the overall resistance of the strain.

One final consideration deserves emphais. At a sufficiently high concentration of penicillin, exceeding the sensitivity of any member of the population, there is no selection. The sensitive and resistant members of the population are apparently killed at the same rate. If the action of these high concentrations is terminated *in vivo* before cure is achieved, or *in vitro* before the culture is sterilized, the surviving bacteria are no more resistant than the original population. As shown in TABLE 1, even if such subcurative treatment is repeated as many as 10 times, the resistance of the final culture is not significantly increased. The provision of high concentrations of penicillin at the focus of infection therefore minimizes the danger, already slight, of developing increased resistance to penicillin. By the same token, however, repository preparations of penicillin, which provide stable concentrations for long periods of time, encourage the selective survival of resistant organisms in the case of those strains which are only incompletely killed at the concentrations provided by these dosage forms. Fortunately, as discussed above, the resistant organisms so obtained differ in sensitivity from the parent strain to so minor a degree as not to be detectable by ordinary methods of assay, and would still be "sensitive" to penicillin in terms of clinical efficacy.

Detailed studies along these lines with aureomycin, chloromycetin, and terramycin have not been reported; but the pattern of resistance with these agents appears to follow that of penicillin rather than streptomycin. Except for staphylococci, there has been no regular increase in the resistance of the organisms commonly treated with these antibiotics and, as in the case of penicillin, there is reason to believe that the antibiotic-resistant staphylococci now encountered may represent the selective multiplication of originally resistant strains, rather than the *de novo* appearance of resistant variants of originally sensitive organisms.

TABLE 1

SHOWING THAT THE PENICILLIN-RESISTANCE OF STREPTOCOCCI AND STAPHYLOCOCCI IS NOT INCREASED BY REPEATED SUBSTERILIZING EXPOSURE TO HIGH CONCENTRATIONS OF PENICILLIN

A. *Group A Streptococci Repeatedly Exposed to Penicillin at 0.1 Micrograms/ml* in Vitro

In each passage, the organisms surviving after 4½ hours at 37° were subcultured in penicillin-free medium preliminary to re-exposure.

Serial passage number	Penicillin resistance of passaged organisms						$\text{LD}_{99.99}$
	% killed after 4½ hrs. exposure to 0.1 µg./ml.	Percent capable of growing out at					
		0.003	0.004	0.006	0.008	0.01	
		micrograms per ml.					
1	99.997	98	0.14	0.00011	<0.00001	<	0.005±
2–12	99.95–<99.99						
13	99.995	100	26	0.00009	0.00027	<	0.005±

B. *Failure of Group A Streptococci to Develop Resistance on Repeated Subcurative Exposure to Penicillin* In Vivo (*Intramuscular Infection in Mice*)

Penicillin treatment			Resistance of bacteria surviving at end of treatment period	
			Concentration (µg./ml.) required to kill	
Salt used	Dosage per injection, mg./kg.	No. of injections	99%	>99.99%
0	0	0	0.006	0.008–0.01
Procaine	2	10	0.006–0.007	0.008–0.01
Sodium	10	4	0.006–0.007	0.008–0.01

C. *Failure of Staphylococci to Develop Resistance on Repeated Substerilizing Exposure to Penicillin at 0.5 Micrograms/ml.* In Vitro, *as Contrasted with the Rapid Development of Resistance at Lower Concentrations*

In each passage, the organisms surviving after 9 hours were subcultured in penicillin-free medium preliminary to re-exposure.

Concn. of penicillin	Passage no.	% killed after 9 hrs.	Percent capable of growing out at						$\text{LD}_{99.99}$
			0.03	0.04	0.06	0.08	0.12	0.16	
0.05	1	99.95	0.7	0.01	0.00016	0	0	—	0.04
	4	0	—	100	50	0.4	0.006	—	0.1
	9	0	—	—	100	70	0.25	0.01	0.16
0.5	1	99.99	0.7	0.01	0.00016	0	0	—	0.04
	5	99.96	0.6	0.002	0.00016	0.00006	0	—	0.04±
	10	99.95	90	3.5	0.0013	0.0003	0	—	0.05±

Conclusion

Although the present review deals with only a few aspects of the problem of drug resistance, it is nevertheless clear that our understanding of the phenomenon, 45 years after its clear delineation by Ehrlich, is still fragmentary.

Indeed, the same could be said of the more general problem, the mode of action of chemotherapeutic agents. In the final elucidation of these problems, a number of different mechanisms will probably be found to be operative, perhaps including some not envisioned by Ehrlich and his co-workers. In large part, however, the broad principles which he evolved, the basic tenet that chemotherapeutic specificity is related to a specific chemical reactivity between the drug and vital cell components, is as valid and pertinent today as it was 45 years ago.

References

1. Demerec, M. 1945. Production of staphylococcus strains resistant to various concentrations of penicillin. Proc. Natl. Acad. Sci. U. S. **31**: 16. M. Demerec. 1948. Origin of bacterial resistance to antibiotics. J. Bact. **56**: 63. M. Demerec. 1949. Patterns of bacterial resistance to penicillin, aureomycin, and streptomycin. J. Clin. Invest. **28**: 891. M. Demerec, E. M. Witkin, B. W. Catlin, J. Flint, W. L. Belser, C. Dissosway, F. L. Kennedy, N. C. Meyer & A. Schwartz. 1949-1950. The gene. Annual Report of the Director of the Department of Genetics. Carnegie Inst. Wash. Year Book. **49**: 144.
2. Lederberg, J. & E. M. Lederberg. 1952. Replica plating and indirect selection of bacterial mutants. J. Bact. **63**: 399.
3. Newcombe, H. B. & R. Hawirko. 1949. Spontaneous mutation to streptomycin resistance and dependence in *Escherichia coli*. J. Bact. **57**: 565. H. B. Newcombe & M. H. Nyholm. 1950. The inheritance of streptomycin resistance and dependence in crosses of *Escherichia coli*. Genetics. **35**: 603. H. B. Newcombe & J. McGregor. 1951. On the nonadaptive nature of change to full streptomycin resistance in *Escherichia coli*. J. Bact. **62**: 539. H. B. Newcombe. 1952. A comparison of spontaneous and induced mutations of *Escherichia coli* to streptomycin resistance and dependence. J. Cellular Comp. Physiol. **39**: 13.
4. Hotchkiss, R. D. 1951. Transfer of penicillin resistance in pneumococci by the desoxyribonucleate derived from resistant cultures. In Genes and Mutations. Cold Spring Harbor Symposia Quant. Biol. **16**: 457.
5. Alexander, H. E. & G. Leidy. 1953. Induction of streptomycin resistance in sensitive *Hemophilus influenzae* by extracts containing desoxyribonucleic acid from the resistant *Hemophilus influenzae*. J. Exptl. Med. **97**: 17.
6. Eagle, H., R. B. Hogan, G. O. Doak & H. G. Steinman. 1944. The therapeutic efficacy of phenylarsenoxides in mouse and rabbit trypanosomiasis (*Tryp. equiperdum*). Public Health Repts. **59**: 765.
7. Hogan, R. B. & H. Eagle. 1944. The pharmacologic basis for the widely varying toxicity of arsenicals. J. Pharmacol. Exptl. Therap. **80**: 93.
8. Ehrlich, P. 1909. Über den jetzigen Stand der Chemotherapie. Ber. **42**: 17. P. Ehrlich. 1913. Chemotherapy. Lancet. **2**: 445.
9. Levaditi, C. 1909. Le mécanisme d'action de dérives arsenicaux dans les trypanosomiases. Ann. inst. Pasteur. **23**: 604.
10. Yorke, W. & F. Murgatroyd. 1930. Studies in chemotherapy. III. The action *in vitro* of certain arsenical and antimonial compounds on *T. rhodesiense* and on atoxyl- and acriflavine-resistant strains of this parasite. Ann. Trop. Med. Parasitol. **24**: 449. W. Yorke, F. Murgatroyd & F. Hawking. 1931. Studies in chemotherapy. V. Preliminary contribution on the nature of drug resistance. Ann. Trop. Med. Parasitol. **25**: 351.
11. Eagle, H. & H. J. Magnuson. 1944. The spontaneous development of arsenic resistance in *Trypanosoma equiperdum* and its mechanism. J. Pharmacol. Exptl. Therap. **82**: 137.
12. Eagle, H. 1954. The binding of penicillin in relation to its cytotoxic action. I. Correlation between the penicillin sensitivity and combining activity of intact bacteria and cell-free extracts. J. Exptl. Med. **99**: 207.
13. Eagle, H. The multiple mechanisms of penicillin resistance. J. Bact. In press.
14. Eagle, H. 1954. The binding of penicillin in relation to its cytotoxic action. III. The binding of penicillin by mammalian cells in tissue culture (HeLa and L strains). J. Exptl. Med. **100**: 117.
15. Ehrlich, P. 1909. Ueber Partialfunktionen der Zelle. Münch. med. Wochschr. **56**: 217.

16. Eagle, H. 1954. The binding of penicillin relation to its cytotoxic action. II. The reactivity with penicillin of resistant variants of streptococci, pneumococci and staphylococci. In press.
17. Eagle, H. The binding of isotopically labeled streptomycin by sensitive, resistant and dependent bacteria. In preparation.
18. Eagle, H., R. Fleischman & M. Levy. 1952. Development of increased bacterial resistance to antibiotics. I. Continuous spectrum of resistance to penicillin, chloramphenicol, and streptomycin. **63**: 623.

VITAL STAINING

By N. Chandler Foot
Department of Anatomy, Cornell University Medical College, New York, N. Y.

The technique of vitally staining experimental animals, a method devised by Ehrlich and his pupils, came into being shortly after the turn of this century and awakened great hopes and aspirations on the part of those who were interested in histogenesis and in experimental histology, pathology, and physiology. Here was a means of staining organs or systems *in vivo*, and it was naturally to be expected that it would far surpass the ordinary histologic methods that were applicable only after the death of the experimental subject. It was, however, soon evident that these promises would not be entirely fulfilled, as the vital stains act specifically only upon cells of one system of the body—the reticuloendothelial—and, to a lesser degree, upon the fibrocytes of connective-tissue.

The technique was widely explored and experimented with, and it was found to be of incalculable value in its restricted field, but once that field had been thoroughly investigated and radioactive isotopes came into general use, the vials of vital dyes began to gather dust on storeroom shelves, and articles dealing with their use dwindled in number. A few years ago, when I had occasion to revise a section in McClung's textbook on histologic methods, I could find nothing recently listed in the Quarterly Cumulative Index and Index Medicus that dealt with the subject except for an article by A. Sorsby (the British ophthalmologist), who published preliminary notes on a method employing dyes of the "light green family." Sorsby discovered that the use of these alkaline dyes would demonstrate damaged cells of neurogenous origin in the retina and published his findings in 1939.[1] The heyday of vital staining was the 1920's, and its years of life were about 20 in number. I accordingly stand before you now in the rôle of an elegist, rather than as an exponent of any novel and up-to-the-minute method. Later on, however, you shall see how important a contribution Paul Ehrlich made to the methods for studying the life and habits of the histiocyte, a contribution that fully justifies discussing vital staining at this meeting.

Before proceeding with this discussion, however, it is necessary to define the term "vital staining." A vital stain is one that can be introduced into a living animal without causing any toxic changes and that, at the same time, will be taken up by, and will stain, certain cells its own peculiar color. The animal continues to live, eats normally, loses no weight, and will even breed and produce young. The term "supravital staining," on the other hand, applies differently. A supravital stain may be employed to dye living cells *in vitro* (as in freshly prepared blood-smears on glass slides), but it is too toxic to be introduced into the body of an animal without causing profound harm. Examples of vital stains are the dyes having a definite color, such as methylene blue, indigo carmine, or the benzidine dyes, all of which will be discussed in due course. Neutral red and Janus green are often combined for the purpose of staining smears of living cells supravitally, and Nile blue and the ammoniated salts of silver will act in a similar fashion. One may also employ colloidal suspensions of metallic or similar material for vital staining, but this procedure is "marking" rather than "staining," as the material is black, or nearly so.

Methylene blue and indigo carmine were used in the latter part of the last century, so that the method is relatively old and has been thoroughly tested. The reason that I was invited to present this paper is the fact that Paul Ehrlich, the genius whom we honor to-day, while experimenting with trypanocidal substances, discovered that there were many dyes of the benzidine family that could be used as vital stains. In 1905, Ehrlich found that trypan red and pyrrhol blue were excellent agents, although the latter was slightly toxic. A year later, Nicolle and Mesnil claimed that another benzidine compound, which they named "trypan blue," was equally efficacious and less toxic than trypan red. Trypan blue replaced most of the other experimental dyes for this reason and was extensively employed between the years 1914 and 1930, after which the method gradually fell into practical disuse.

In 1909, Ehrlich[2] was invited to address the German Chemical Society on the subject of the status of chemotherapy as of that date. His paper dealt chiefly with arsenical antisyphilitic remedies, but he also referred in passing to his work on vital staining as a means of determining the distribution of chemical substances introduced into the living organism. He described the specific affinity of methylene blue for the nervous tissue and its tendency to stain cells rather diffusely. He contrasted with this tendency the affinity of neutral red and pyrrhol blue for the vital granules of Flemming in certain cells, and he enthusiastically commended the work of E. E. Goldmann who, in two long articles[3, 4] on vital staining, later expressed with equal fervor his deep indebtedness to Professor Ehrlich for putting at his disposal his vast knowledge of vital dyes, as well as his voluminous stocks of the dyes themselves. I am old enough to have known men who worked in Ehrlich's laboratory, and they described to me his habit of hoarding bottle after bottle of new chemical substances and dyes as they came to him from the manufacturers, and of testing them out *seriatim* in experiments to establish their value, or their lack of it. The benzidine dyes with which he experimented bore the consecutive numbers of the 1800 series, which will give some idea of the vastness of chemical material that was covered in his laboratory.

Naturally enough, he could not do everything personally, and it was Goldmann, then, who took over the task of working out the possibilities of these benzidine stains, while Ehrlich continued to pursue his therapeutic problems. Goldmann's papers were long, meticulous, and decidedly dry reading. Furthermore, they appeared in a surgical journal, and this circumstance accounts for the fact that they were "buried" and comparatively unknown to the histologists and pathologists. The surgeons were not overmuch interested in them, and the laboratory men did not anticipate finding an article of this sort in a surgical publication. An American histologist, H. M. Evans,[5] worked in Ehrlich's laboratory on the benzidine dyes and their applications. When he returned to the United States, he brought with him some of Ehrlich's stock to experiment with, publishing some excellent papers which *were* read by histologists and pathologists, and these papers stimulated much work along the line of vital staining. One of Evans' publications, written in collaboration with Werner Schulemann (who apparently constituted the chemical and relatively silent partner in the team), appeared in *Science* and attracted much attention. It gave a long description of the chemical interrelationship of the benzidine compounds and is well worth reading by those whose interests lie along such lines.

Concurrently with the use of the benzidine dyes, particulate material in the form of colloidal suspensions of carbon, lithium carmine, and like substances were tried out and found to be excellent for investigating that certain type of cell which we have already mentioned—the histiocyte. McJunkin[6] was one of the first to use carbon, preparing his own suspensions from lampblack and water containing 2 per cent gelatine. It was found by someone, whose identity I have never been able to ascertain, that Higgins' Waterproof India Ink could be used just as safely and much more conveniently. This ink was recommended to me in 1921 by Professor George Wislocki of the Harvard Medical School, who had learned of its convenience while he was at Johns Hopkins. Ehrlich referred to indigo carmine in the address to the Chemical Society already quoted, but apparently he used it relatively little, preferring the benzidine compounds.

The use of colloidal suspensions of metal was another approach to the subject. Lund, Shaw, and Drinker[7] employed suspensions of finely particulate manganese dioxide for studying the distribution of foreign material in the body. Instead of the microscope, they resorted to chemical assays of the manganese contained in the various organs and systems. This procedure is a chemical variant of the technique of vital staining that has been used with success.

As we are chiefly interested in Ehrlich's connection with vital staining, we now go on to an exposition of the method and a consideration of the essential chemistry of the benzidine dyes, the application of which was his discovery and one of his many contributions to experimental medicine. Although Ehrlich recognized the importance of methylene blue and neutral red, his real interest was centered in the benzidine group.

The first member of the family to be used was trypan red, the formula of which is illustrated in FIGURE 1. It was found that this dye underwent little

Trypan Red

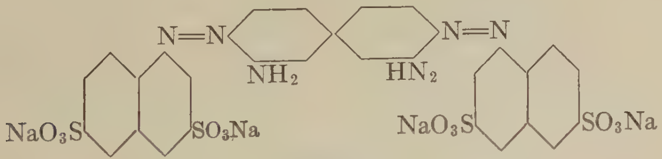

FIGURE 1

if any change in the body and was "completely nontoxic." Ehrlich used it extensively in his experiments in 1905 and afterward, before turning over further investigation to his assistants. The latter concentrated on the question of the chemistry and the mechanism of staining as applied to this group. In 1906, Nicolle and Mesnil (quoted by Evans and Schulemann[4]) found another benzidine dye which they named "trypan blue," the formula of which is shown in FIGURE 2. There is a rearrangement of the grouping of the elements, but little "new has been added" save for a couple of hydroxyl groups and a shift in the position of the double-bonded nitrogen molecules. The close similarity of the two formulae will be at once evident.

Trypan Blue

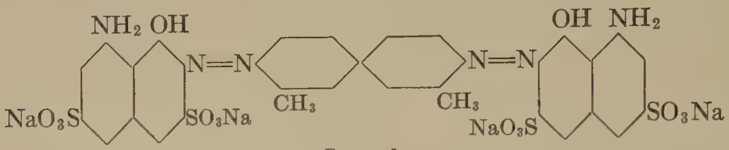

FIGURE 2

If 1 cc. of a 0.5 per cent solution of either of these dyes be injected into an ear vein of a rabbit, the animal will begin to take on the red or blue color of the compound used within a few minutes. There will be a progressive coloration of the ears, tip of the nose, tail, and mucous membranes (most evident in the tongue and oral mucosa). This effect is shortly followed by a similar change in the color of the skin as a whole. A Kodachrome photograph was taken 72 hours after beginning the injections. This animal had an initial intravenous dose of 1 cc. of 1 per cent aqueous trypan blue into an ear vein, followed by two intraperitoneal injections of 5 cc. each of 1 per cent solution of the same dye. With successive doses, the color becomes more and more intense, the animal secretes red or green urine and, if lactating, red or blue milk. To quote Evans and Schulemann, "The animal thus stained plays, eats, breeds, and in all ways manifests its normal activity and there is no evidence of the ill-effect of the dye."

Microscopic investigation of the tissues of such animals will show that the pigment is largely contained in the phagocytic histiocytes of the body. Fibroblasts take it up more slowly and in the form of smaller granules or globules, but other tissues show little or no affinity for it. The epithelium of the liver and convoluted renal tubules (concerned with getting rid of the dye) become progressively and diffusely stained as administration is continued.

The histiocytes, which are known by a long list of aliases ("endothelial phagocytes," "dust-cells," "heart-failure cells," "clasmatocytes," "macrophages," "cellules rhagiocrines," *etc.*) take up the dye in a specific way. They are not diffusely stained, and the nucleus has not the slightest affinity for the stain. It is the "vital granules" of Flemming which attract the colored molecules of the dye. Should a cell die off before the stain is administered, however, it will take on a diffuse coloration and its nucleus will be precisely stained. This effect is exactly what we also note in supravitally stained smears. The phenomenon has its applications in cases where one wishes to distinguish dead from living cells, as in certain necrobiotic processes.

Having ascertained these facts, Ehrlich's co-workers were quite naturally intrigued by the question of solving the mechanics of the process. Why did this comparatively segregated group of cells take up the dye and why were they almost the only ones to do so? We can dismiss the diffuse tinging of the epithelium of the liver cords and convoluted renal tubules by smugly asserting that, as they are drenched with the dye, and as it is their function to excrete it, they become diffusely impregnated with it. After the dye has been excreted they pale off, but the granules of the histiocytes and fibroblasts may retain the color for months. Ehrlich attributed this phenomenon to the presence of receptors in these granules, bonds having an affinity for a certain chemical group within the benzidine molecule.

Overton (quoted by Evans and Schulemann) took the view that vital staining depended upon the solution of the dyes in fats or lipids. This idea may be supported by the fact that methylene blue and neutral red are both fat-soluble and that the Flemming granules contain lipids which might act as solvents, but it fails to explain why the benzidine dyes, which are insoluble in fat, should be taken up by these same granules.

Ehrlich's collaborators found that not all benzidine dyes stain vitally. For example, if they administered azo blue, which has the formula shown in FIGURE 3 and closely resembles trypan blue in its structure, they found, days later, that the injected dye was still concentrated at the point of application under the skin, and that there was no evidence that it had diffused to produce any vital staining. As Ehrlich had postulated that the alpha naphthol group might be the ceptor, Evans and Schulemann explored this possibility without success. Many vital dyes which gave excellent results lacked this group and yet they stained. Thwarted in this approach, they next searched for some characteristic which would be common to the vital benzidine stains. Most of the efficient dyes contain disulfonic acid groups, this radicle apparently exerting a favorable effect by rendering the dye more diffusible and rapidly distributed. They found, however, that supersulfonation of benzidine compounds resulted not in the expected increase in diffusibility, distribution, or "take-up" on the way *into* the organism, but rather in an unexpected increase in the rate of excretion of the substance on the way *out* of it. The output was accelerated and the state of vital staining became a very transitory affair.

Azo Blue

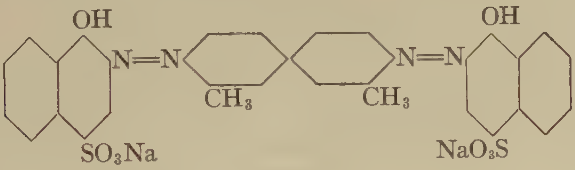

FIGURE 3

Benzopurpurine B can be injected in the form of a freshly prepared cold suspension, and it will stain vitally, but if it be permitted to stand about in the laboratory for a day or two, administration of the same suspension now produces almost instantaneous death from cerebral embolism. The formula of this benzopurpurine is immaterial, but it resembles those of the other benzidine dyes quite closely. The phenomenon just described is supposed to depend upon the production of larger molecules, or coarser aggregates of molecules, too bulky to pass the finer capillaries of the cerebrum. It is apparently a matter of electrocoagulation, as the use of normal saline or Ringer's solution as a suspending fluid for any benzidine dye invariably produces fatal effects, even when a freshly-prepared suspension is injected. Distilled water must therefore always be used for this purpose.

This development brings up the matter of the size of the colloid molecules in the suspension as a factor in vital staining, which (presumably out of loyalty to Ehrlich) Evans and Schulemann held in reserve for a dramatic climax in their discussion of the various theories. At long last, they took up the matter of the absorption of colloid particles by the histiocytes, including carbon, metallic silver, and other metals. These materials act precisely like the vital stains of the benzidine group (albeit somewhat more slowly), yet they have no elaborate chemical structure, being simply metallic elements suspended in a menstruum that will render them colloidal, such as gelatinized water, or water with an emulsifying agent such as gum acacia. Also, they have no color other than plain black, if we except the carmine group. Therefore the authors hinted at phagocytosis as an explanation for vital staining, which introduces another problem: just what is involved in the process of phagocytosis? Metchnikoff investigated this question very thoroughly and he has had a large following of eager researchers in this field. To condense the various theories, we fall back upon diffusibility, the size of the particle to be phagocytosed, "affinity" (which is another very provocative term that suggests further explanation and is, incidentally, useful if vague), stickiness of the molecule and, lastly, simple mechanics such as the ameboid engulfment of the material by the cytoplasm of the cell. Once inside the cellular membrane, the question of how the molecule becomes concentrated in the vital granule arises to plague us further.

When the various papers already referred to were published, they caused a great furor, and histologists, both of the normal and pathologic variety, began to experiment with the method. This method was a means of staining cells *in vivo* and *in situ* as well without resorting to death and fixation. The technique offered a rosy (or at least polychrome) future, according to the dyes used. It soon became evident, however, that this apparently limitless ceiling for staining *in vivo* was, in sober fact, much lower than estimated. The stains did not affect the epithelium (with the already noted exceptions of liver and renal cortex). They did not even tinge the nervous system, nor the bony skeleton, nor cartilage. Sorsby's light green stained only damaged and presumably dying cells. The use of these stains was thus limited to that fortunately enormous and rather poorly understood family of cells that Aschoff later christened "histiocytes"; cells that are peculiar in that they have acquired an entire vocabulary of names, appellations based in the main upon their usual menu or diet. Should they ingest fat they are "lipophages." If their diet be hemosiderin, they are then "Herzfehlerzellen" (frequently mistranslated "heart-failure cells" instead of "heart-defect cells," which this term really means). Should they become enlarged and comparatively dense, they are "epithelioid cells" of tuberculous and other granulomas. When foamy and reticulated, they are "lepra cells" in leprosy, or "Gitterzellen" in German neuropathology. It happens that, at the time of the introduction of vital staining, there was much confusion concerning these ubiquitous scavengers, and it was by applying the new method to experimental procedures in animals that much was learned concerning their origin and propagation. Supravital stains had about played their part. Vital stains carried on the work to the point where it became evident that these multifarious cells were, in reality, merely one and the same element performing various functions under a variety of dietary disguises and aliases. If for no other reason, then, we can claim a great deal of credit for the technique of vital staining and for the man who perfected it, Paul Ehrlich.

Ehrlich's antisyphilitic remedies, salvarsan and its less toxic progeny or successors, played a very important rôle in therapeutics up to the advent of the far less toxic antibiotics such as penicillin. Then, of course, these earlier remedies of his were completely superseded by the antibiotics. Similarly, vital staining was important in clearing up much of the confusion that surrounded the histiocyte, with its innumerable aliases. Vital staining has now yielded to other methods of identifying cells, notably that of impregnating them with radioactive substances which can be traced on the photographic film in "autoradiographs" or detected by the clicking of the Geiger counter. Consequently, there has been this comparative dearth of publications on the subject of vital staining to which I have already referred. Apparently vital staining has seen its best days and is now in retirement, like so many of the men who experimented with it in the torrid twenties. We are therefore glad that we had the use of this method. We feel that we employed it to good advantage, but we are now aware of its shortcomings.

Some of the important uses to which vital staining has been put are: the just discussed matter of the identity and origin of the histiocyte; the physiology of the absorption of appropriately stained amniotic fluid;[8] the distribution of marrow in forming bones;[9] and the fate of particulate foreign material in the body.[7] Wislocki was responsible for the papers on amniotic fluid and bone marrow distribution, and he also discovered a tiny, vitally stainable area in the otherwise refractory nervous tissue of the midbrain of experimental animals, an area in which many histiocytes had congregated. His method for demonstrating bone marrow was a combination of the Spalteholtz technique for rendering bone transparent with toluene, plus vitally staining the marrow with Higgins' Ink. The jet-black marrow was clearly visible through the amber-yellow bone. Several of us have studied the changed distribution of colloidal carbon following splenectomy. The spleen usually filters out most of the foreign matter that gets into the circulation, and its removal will throw the onus for this task upon the capillary beds of the lung, liver, and lymph nodes, which must compensate for the lack of the splenic filter. The list of similar applications of vital staining to experimental physiology, pathology, and medicine could be much expanded.

As to the *modus operandi* of vital staining, let us take up the particulate dyes first. Higgins' Ink does not have to be sterilized. One dilutes it with equal parts of sterile distilled water and injects it into a convenient vein. Five cc. may be given the average adult rabbit daily for three or four days. When using lithium carmine, the stain preferred by Aschoff and Kiyono, it should not be administered to Japanese waltzing mice, a strain unpleasantly susceptible to it. Colloidal suspensions of silver and gold have been used but, aside from their alluring costliness, there appears to be little to recommend them over cheaper methods.

As for the benzidine dyes, the least toxic are the two "trypans"—red or blue—of which the blue has always been preferred to the red on account of its supposed lesser toxicity. Personally, I have found them equally safe and efficient, and have chosen them for use as one would choose from a palette, using the color most suitable for the purpose of the experiment in hand. For instance, if tubercle bacilli are being stained in the sections, one would never use trypan red, as it would be too readily confused with carbol fuchsin. Trypan blue was manufactured in Buffalo during the first World War under the name of "Niagara blue." I found it as effective as the Grübler trypan blue.

For injection, a fresh 0.5 per cent suspension is prepared in distilled water, and one or more cc. is introduced into whatever portion of the experimental animal seems best indicated to produce the best results. The suspension should be sterilized by boiling and allowed to cool. For rapid staining, the circulation *via* intravenous injection is best. For slower absorption over longer periods of time, a larger dose (5 cc. or more) may be administered intraperitoneally. Subcutaneous injection is also a possible avenue of approach, but still slower as to results.

So when Ehrlich started the work on the benzidine dyes, he aided and abetted an era of medical investigation that involved many men who either won reputations by carrying out suggestions he had made, or enhanced merit they had already acquired. We owe Ehrlich a deep debt of gratitude for this development and for his many other contributions to medicine. Through the use of vital dyes, Aschoff[10] arrived at the concept of the "reticuloendothelial system," and the mystery of the cellular unit of this system was thereby largely cleared away. H. M. Evans, Wislocki, Winternitz, Maximow, Bloom, and a host of other workers in the field of histology or histopathology have all employed vital staining of one type or another in their attack upon a wide variety of problems. If vital staining is at last defunct, let us offer a devout "*requiescat in pace!*"

References

1. Sorsby, A. 1939. Vital staining of retina—a preliminary note. Brit. J. Ophthalmol. **23**: 20.
2. Ehrlich, P. 1909. Ueber den jetzigen Stand der Chemotherapie. Ber. **42**: 17.
3. Goldmann, E. E. 1909. Die innere und äussere Sekretion des gesunden and kranken Organismus im Lichte der vitalen Färbung. I. Beitr. klin. Chir. Bruns' **64**: 192.
4. Goldmann, E. E. 1914. Die innere und äussere Sekretion des gesunden und kranken Organismus im Lichte der vitalen Färbung. II. Beitr. klin. Chir. Bruns'. **78**: 1.
5. Evans, H. M. & W. Schulemann. 1914. The action of vital stains belonging to the benzidine group. Science. (n. s.) **39**: 443.
6. McJunkin, F. A. 1918. Simple technic for the demonstration of a phagocytic mononuclear cell in the circulating blood. Arch. Internal Med. **21**: 59.

7. Lund, C. C., L. A. Shaw & C. K. Drinker. 1921. Quantitative distribution of particulate material (manganese dioxide) administered intravenously to the dog, rabbit, guinea pig, rat, chicken and turtle. J. Exptl. Med. **33** : 231.
8. Wislocki, G. B. 1921. The fate of true solutions (phenolsulphone-phthalein) and colloids (trypan blue) injected into the mammalian embryo. Bull. Johns Hopkins Hosp. **32** : 93.
9. Wislocki, G. B. 1921. Experimental observations on bone marrow. Bull. Johns Hopkins Hosp. **32** : 138.
10. Aschoff, L. 1924. The Reticulo-Endothelial System. Lectures on Pathology. Hoeber. New York.

BLOOD MORPHOLOGY

By Paul Reznikoff
Department of Medicine, New York Hospital and Cornell University Medical College, New York, N. Y.

Modern hematology may be said to have had its origin with the work of Paul Ehrlich. It is therefore particularly pertinent to introduce this subject of blood morphology, with a brief itemization of this great scientist's contributions to the study of blood.

In 1880[1] Ehrlich published his epoch-making paper, describing the use of his triacid solution in staining thin blood films. This development permitted the differentiation of the formed elements of the blood as we know them today. Ehrlich was the fist to recognize stippling or punctate basophilia in the red blood cell.[2] He pointed to the changes in the bone marrow in pernicious anemia,[3] and reported the earliest case of aplastic anemia.[4] While a student in 1877[5] Ehrlich clarified the status of the mast cell or basophile.[6] These are only a few of the many important studies of Paul Ehrlich.

The formed elements of the normal blood are the following: red blood cells; white blood cells, consisting of polymorphonuclear cells or neutrophiles, lymphocytes, monocytes, eosinophiles, and basophiles; and the thrombocytes or platelets. In this paper, the quantitative distribution of the cells, their origin in the blood-forming organs, their functions or their pathological variations will not be discussed and, for the most part, only the human adult cells will be the concern of this report.

The Red Blood Cell

The mature red blood cells are nonnucleated, biconcave discs, measuring 7 to 8.5 micra in diameter. They arise in the sinuses of the bone marrow and, as their maturation takes place, the erythrocytes reach the circulation and are washed into the blood stream.

The precursors of the red blood cells are nucleated and lack hemoglobin. The early normal progenitor is called an erythroblast and, with development and the acquisition of hemoglobin, the nucleus shrinks, becomes pycnotic and the cell is now called a normoblast. The nucleus disappears either by extrusion or lysis. Between the stage of the nucleated and the mature cell, a fine reticulum may be demonstrated with special stains such as brilliant cresyl blue. These reticulocytes have an important significance in judging the activity of red blood cell production since they are increased in number (above 2 per cent of the cells in the peripheral blood) when the bone marrow is stimulated by such conditions as hemorrhage, hemolysis (destruction of cells in the circulation), and stimulation in anemia by iron or vitamin B_{12}.

With ordinary staining methods or when fresh blood is examined by the usual microscopic methods, no structure is demonstrable in the red blood cell. The cell changes its shape readily as it is squeezed through the capillary circulation and appears to be a homogeneous bag filled with a hemoglobin-containing fluid. The function of the hemoglobin is to carry oxygen to the cells.

When the red blood cells are exposed to the air, they change their shape and become crenated. This change is characterized by a bumpy appearance of the surface.

In abnormal states, the erythrocyte may show many bizarre appearances. In certain disorders, the cells show marked variations in shape (poikilocytosis) or in size (anisocytosis). In hemolytic diseases, the cells become spheroid (spherocytosis). In sickle cell anemia, deprivation of oxygen produces a sickle shape. Recently Singer and his associates[7] described an interesting condition which they call acanthrocytosis, consisting of marked deformity of the red cells showing several coarse projections, a state they think is genetically conditioned.

Also with special stains, certain structures are seen in the cells such as Howell-Jolly bodies, Cabot rings, punctate basophila or stippling seen in lead poisoning, and Heinz bodies in hemolysis.

A detailed account of such morphological variations has been described by Isaacs.[8]

Another type of structural study of the red blood cell is afforded by the hematocrit determination. This method consists of centrifuging blood in a standard tube and manner. Ordinarily, about 45 per cent of the blood is composed of red blood cells after centrifugalization. By comparing the hematocrit reading with the red blood cell count, a value called the mean corpuscular volume is obtained which permits a conclusion as to whether the cells are of normal (normocytosis), small (microcytosis), or large (macrocytosis) volume. In iron deficiency and Mediterranean anemias, microcytosis is found. In pernicious anemia, macrocytosis is usual.

The diameter of the cell may be measured by direct microscopy (the Price-Jones curve) or by projecting the erythrocytes upon a screen under conditions which permit known enlargement.

The development of the electron microscope has permitted investigators to throw new light on the structure of the erythrocyte.

Bernhard,[9] by electron microscopic studies, finds that the intact red blood cell has an extremely dense internal structure of granular or filamentous appearance when fixed with osmic acid. Striking orderliness in architecture is present. A spongy network and a well-defined membrane appear only when hemolysis sets in. The completely hemolysed erythrocyte is empty and balloon-like. Bloch and Powell[10] state that red blood cells have surfaces of sharp outline and that when blood is sludged irregular surfaces are seen with an apparent coating.

Sunderharagiati and Wright[11] report that normal erythrocyte membranes show finely granular surfaces, and that only rarely are holes or fold formations seen. But in many disorders of the blood, increased coarseness, holes, and fold formations appear. These fold formations may be reproduced experimentally with chemical agents. Irradiated erythrocytes show no changes, nor do stored cells.

One of the most detailed reviews of the architecture of the red blood cell is that of Ponder.[12] He enumerates the known constituents of the cell and indicates their probable location. The fixed framework of the cell consists of

3.4 per cent by dry weight of the entire erythrocyte. The surface is between 150A and 250A thick, and 40 to 60 per cent of the lipid is bound to protein. Electrophoretic data indicate that the surface is dominated by strong acidic groups. This surface is not composed of protein alone, but also of lipid. The most striking feature of the cell interior is the intimacy with which the hemoglobin molecules are situated with respect to each other. Ponder states that about 63 per cent of the cell consists of water. Other constituents are hemoglobin, sodium, potassium, chloride, bicarbonate, lipid, phospholipid, neutral fat, cerebrosides, cholesterol, nonhemoglobin protein, sugar, lecithin, phosphates, nonprotein nitrogen, creatin, creatinine, and sulfates. Also in the cell are found glutathione, adenine nucleotides, and enzymes. In the March 1954 issue of *Blood*, Ponder presented an analytical review of our present concepts of the mammalian red blood cell which clarifies many of the problems concerned with the erythrocyte.

The phase-contrast microscope and dark field studies have also thrown light on the structure of the erythrocyte.[13, 14]

Finally, cytochemistry has contributed important data for our understanding of the constituents and structure of the erythrocyte and indeed of all blood cells.

Granick,[15] in a comprehensive report, has listed the constituents of the erythrocyte and suggests from a chemical viewpoint how the red blood cell develops and functions.

Ralph[16] has employed supravital stains and Sudan black B, and he indicates that the erythrocyte has a plasmn membrane which has the following characteristics: it is acted upon by plasma enzymes, and may be jelled by citrates and oxalates. This membrane contains alcohol and acetone extractable lipids which cannot be stained in the natural state but which can be stained with Sudan black after fixation. Hemolysis does not cause any visible rupture of the plasma membrane. The mature normal erythrocyte has no visible internal structure but the immature cell may contain mitochondria, granules, and reticulum. He also found that the thickness and refractivity of the surface in the dark field is directly proportional to the concentration of contained hemoglobin.

An interesting contribution by Bloom and Wislocki,[17] using Baker's method of staining, indicates that phospholipids rarely are found in mature red blood cells but are seen in immature erythrocytes.

The White Blood Cells

The neutrophile (polymorphonuclear cell) varies between 9 to 12 micra in diameter and has a cytoplasm and nucleus. The cytoplasm has an outer clear protoplasmic zone (a hyaline exoplasm) and a granular endoplasm. The pseudopodia are formed primarily by a projection of this exoplasm and the endoplasm with its granules flows into this outthrust. The granules do not appear to enter the ectoplasm. The cytoplasm contains centrosomes and a centrosphere, mitochondria, Golgi apparatus, and vacuoles. In addition, the neutrophile is full of refractile granules which may be stained with a neutral dye: hence the name of the cell, neutrophile. Bunting,[18] reviewing the literature on the subject, states that these granules probably carry enzymes or enzyme-like catalyzers and may consist chiefly of lipids. It has recently been suggested that toxic granulation, a condition occurring in infections, where the granules become large and irregular, is similar to cloudy swelling seen in other organs in disease.[19]

The nucleus of the polymorphonuclear cell is unique in the marked variability of its shape. It is generally believed that in its early stage it is round, and that as the cell develops the nucleus indents, becomes band-shaped, and eventually divides into several lobes joined together by narrow nuclear material which may be threadlike in thinness. This characteristic, described by Arneth and by Schilling, is used to study the so-called shift to the left or degree of immaturity of the neutrophile in many clinical conditions such as infections, myocardial infarcts and, in general, wherever tissue damage is found.

Kautz and DeMarch[20] studied sectioned neutrophiles with the electron microscope and found the nucleus to be relatively homogeneous and the nuclear membrane thin. The cytoplasm is finely granular and its electron scattering power less than that of the nucleus. The cytoplasm contains occasional vacuoles. The granules are homogeneous and dense with clearly defined membranes. Their diameter ranges between 0.1 and 0.25 micra. The smallest granules may be a second species of granules or mitochondria. True mitochondria are not observed.

The living lymphocyte, according to Bloom,[21] measures 6 to 8 micra in diameter and in fixed smears varies from 7 to 10 micra. This cell contains relatively little cytoplasm, the nucleus filling almost the entire cell. In the large lymphocyte, more cytoplasmic substance is found. In the cytoplasm are a variable number of azurophilic granules, and the nucleus is usually round but may be slightly indented. It was once thought that the lymphocyte could not move but, by tissue culture technique and in supravital studies, it can be seen that the nucleus seems to advance first and the cytoplasm seems to be pushed or drawn out. Supravital staining also indicates the presence of mitochondria and vacuoles in the cytoplasm.

In many of the descriptions of the lymphocytes it has been believed that large lymphocytes with more cytoplasm have a special significance but no good evidence has been presented that small and large lymphocytes have different origins or functions. Also it was believed that young lymphocytes have darker cytoplasms but this concept has not been verified.

Electron microscopic studies[20] show that both the cytoplasm and the nucleus of the lymphocytes possess much greater electron scattering power than of any other blood cell. The nuclear material is clumped in a coarse network, especially along the nuclear membrane, and an indentation of the nucleus is often seen. The cytoplasm is also dense and rather finely precipitated. The mitochondria appear to be elongated rods of a striped appearance. Two types of mitrochondria exist: (1) most of the filamentous type; (2) the small and rounded type.

Recently, Petrakis[22] used a microspectrophotometric method to estimate the desoxyribonucleic acid in lymphocytes and showed that he could correlate the quantity of this substance in the cell with the chromosome number.

Cytochemical studies have thrown light upon structures in the cell. Hempelmann and Knowlton[23] studied the refractive index and the birefringence of the nonazurophilic granules. They also used microspectrophotometry, and came to the conclusion that these granules are composed of lipid material, probably phospholipids.

The monocyte is a mononuclear cell measuring about 10 to 11 micra in diameter. It is derived from the reticuloendothelial system[21] and is frequently confused with the lymphocyte, although in human blood these two cells are not difficult to distinguish from one antoher. The nucleus takes a lighter stain than does that of the lymphocyte, and the cytoplasm is more abundant, bluish with the ordinary stains and diffusely granular. Mitochondria and vacuoles are seen in the cytoplasm. When stained with neutral red, a rosette of red vacuoles about the cytocentrum has been described by several workers as the chief identifying characteristic of the monocyte, but others believe that many types of cells, including the lymphocytes, may show this feature, especially in animals.

Kautz and DeMarch[20] found by the electron microscope method that the monocyte is only slightly smaller than the largest sectioned neutrophile. The nucleus has an electron density similar to that of the neutrophile and, in some of their observations, the double nature of the nuclear membrane is evident. Many of the nuclei are indented. With their fixation method, the cytoplasmic material seemed precipitated in a rather sparse network. Vacuoles are seen which are either vacant or contain structures which probably are mitochondria. The mitochondria have a well-defined membrane and exhibit pairs of filamentous structures running perpendicular to the long axis of the mitochondrium.

Eosinophiles measure about 9 micra in diameter in the fresh state and 12 micra in dry preparations.[24] The nucleus is round and bilobed, rarely trilobed, and the lobes usually present a symmetrical appearance. The chief characteristic of the cell is the cytoplasm, which is filled with large refractile granules which have a great affinity for acid dyes. In the fresh state, the granules have a yellowish or greenish color and, under dark field illumination, take on a golden yellow hue. With neutral red they also stain golden yellow. The nature of the eosinophilic granules is not clear, but the contention that they represent phagocytized hemoglobin or its dissociation products is not generally accepted. The granules give a positive peroxidase reaction, are soluble in concentrated acids, alkalies, and warm acetic acid, give a xanthoproteic reaction, and contain iron. They may be combinations of proteins and lipids. The striking feature of the eosinophile is its increase in number in allergic conditions, parasitic infections, skin disorders, and in polyarteritis nodosa. All the latter diseases may of course cause eosinophilia by sensitization.

Electron microscopic studies[20] indicate that the granules of eosinophiles measure about 0.5 micron and have two components: a very dense central substance possessing extremely high electron scattering power, and a lighter surrounding portion. A limiting membrane is not clearly seen. Mitochondria are not seen. A nucleolus represented by a dense central spot is present.

Cytochemical studies by Wislocki, Bunting, and Dempsey[25] indicate that eosinophiles, at least in the tissue, contain no iron and that their granules show no acid or alkaline glycerophosphatases.

Less is known about the basophilic leukocyte than about the other white blood cells because of their paucity (usually less than 50 per c. mm.), and because their function is unknown.[26] The basophile averages 8 to 10 micra in diameter, the nucleus is polymorphous, although this has been questioned, and the cytoplasm contains large granules which are stained with basic dyes. These granules are nonrefractive and are not as uniformly distributed nor of the same volume and form as the granules of eosinophiles. The granules are water soluble. The cytoplasm is oxyphilic and homogeneous. Ameboid motion is slower than in other granulocytes. Whether the basophile has phagocytic properties is not settled. Also there is a difference of opinion about the peroxidase reaction in this cell. Some workers maintain that the basophile is an "unripe" or degenerated eosinophile but there is no evidence for such a theory. Ehrlich[27] showed that the basophile is a normal constituent of the bone marrow and therefore of the blood, and that it is independent of the eosinophile and lymphocyte. The basophiles are increased in such pathological conditions as myelocytic leukemia, polycythemia, neoplasms involving the bone marrow, and in some cases of agnogenic metaplasia.

Electron microscopic studies[20] indicate that the granules of the basophile are about one micron long and show a distinct membrane. Droplets which appear fatty and striped mitochondria are also seen. Wislocki, Bunting, and Dempsey[25] showed by their cytochemical methods that tissue basophiles contain both acid and alkaline glycerophosphatases.

The Thrombocytes (platelets)[28]

Blood platelets or thrombocytes are irregular or rounded shaped objects measuring about two to four micra in diameter and are isolated or clumped in a blood smear. With the usual polychrome stains, the central portion appears dark reddish violet and granular, and the surrounding portion looks light blue and homogeneous. In pathological conditions such as leukemia, platelets appear swollen or enlarged, and even parts of their precursors or megakaryocytes may be seen.

In fresh unstained preparations, platelets assume an oval or elongated appearance. With supravital stains such as neutral red, platelets take up the color of the dye, and, with Janus green, mitochondria are seen. Fat globules have also been reported in some thrombocytes.

When anticoagulants are used, platelets have a greenish fluorescent appearance. Thrombocytes show Brownian movement in citrate solution.

Of all the formed elements, platelets break up most readily, disappearing within a few hours unless the blood is drawn by special methods, using Arquad 2-C for coating needles and silicone for coating glassware. Recently a new anticoagulant, Sequesterene disodium, has been found to be of considerable value in preventing agglutination and disintegration of platelets.[29]

While it is not within the province of this paper to describe the bone marrow and lymphoid precursors of the blood cells, it is of interest to mention some characteristics of megakaryocytes from which platelets are derived. These structures are very large, measuring 35 to 40 micra in diameter. The nucleus occupies a considerable portion of the cell and is multilobed, stains deep purple, and contains nucleoli. The cytoplasm is finely granular and stains purplish red. The peripheral zone presents a bluish hyaline appearance. The megakaryocyte may be round or irregular and may have pseudopods. Schwarz[30] has demonstrated that the development of granules in the megakaryocyte begins in a limited area located near the indentation of the nucleus and by concentric, continuous expansion granulopoiesis involves the entire cytoplasm except a narrow border zone. This movement keeps the nucleus in an eccentric position and the structure does not partake in the formation of granules.

Pisciotta, Stefanini, and Dameshek[31] have thrown some interesting light on normal megakaryocytes and on those found in idiopathic thrombocytopenic purpura, using phase-contrast microscopy. Wislocki and his co-workers[25] showed that basophilia of megakaryocytes in certain animals is abolished if the cells are exposed to ribonuclease, indicating that the cytoplasm contains ribonucleoprotein. They believe that their cytochemical studies indicate that platelets arise from the cytoplasm, not from the nucleus.

Braunsteiner, Fellinger, and Pakesch[32] found by electron microscopy that the plasma cell contains a filamentous structure which is probably a ribonucleoprotein.

Supravital staining of the blood cell has created much interest in the past.[33, 34] By the use of stains such as neutral red and Janus green, the individual cells could be distinguished by virtue of the appearance of the granules. The method was used in a warm box by most workers and permitted especially the differentiation between lymphocytes and monocytes. However, Hall[35] has shown that, except for very rare cases, the supravital technique has no advantage over the simple dry-smear method. Schwind,[36] however, stated that, if new techniques and stains are used, information of much value can be obtained by this method.

The peroxidase stains merit mention in differentiating the various leukocytes. In myeloid cells, transference of oxygen from the air, or using hydrogen peroxide as a source of oxygen, permits an oxidizing ferment in the neutrophiles and eosinophiles to deposit benzidine as large granules within the cell. Using the Goodpasture method or the Beacom modification results in deep blue granules in myelocytes, polymorphonuclears, or eosinophiles. Monocytes are distinguished by fine granules, and lymphocytes deposit none. The method of Sato and Sekiya is used in many laboratories with much success. It is important to stain a normal smear as a control with the peroxidase technique.[37]

Finally, it is of interest to note the information which has been made available by cytochemical studies by various authors. Some of the additions to our knowledge have already been mentioned, and only a few examples of this new and promising method of investigation can be presented in this report.

Wislocki, Rheingold, and Dempsey[38] have determined the glycogen content of the blood cells by the periodic acid-Schiff reaction. From the same laboratory,[39] an excellent review of this important field was published which summarized our knowledge of histochemistry applied to hematology up to 1948.

Wachstein[40] found a reducing activity in blood cell granules, using 3,5 diphenyl tetrazolium chloride.

Bloom and Wislocki[41] studied the distribution of phospholipids in cells, using the Baker method and Sudan black B stains.

Kirkman[42] found globules in many cells including those from the blood which contain a mucopolysaccharide, poor in free aldehyde groups, combined with a strongly basic protein.

Ayres and Starkey[43] used cytochemical, crystallographic, and phase microscopic methods to show that Charcot-Leyden crystals are crystalline proteins derived only from the nucleus of eosinophiles.

This brief review indicates that the structure of the cell is complex and that its study has often been barren of significance, because too much attention has been paid to a description of minutiae, and functional interpretation of observations has not been sought or perhaps has not been possible. Paul Ehrlich founded modern hematology by adding imagination to his keen observations and by recognizing the importance of applying biological meaning to descriptions which, by themselves alone, are scientifically sterile. Let us hope that the newer methods of hematologic study, such as electron microscopy and histochemistry, will give a purpose and an interest to the rather lifeless subject of purely descriptive morphology.

References

1. Ehrlich, P. 1880. Z. klin. Med. **1**: 553.
2. Ehrlich, P. 1885. Charité-Annalen. **10**: 136.
3. Ehrlich, P. & A. Lazarus. 1898. Normale und pathologische Histologie des Blutes Abteilung 1. Holden. Vienna, Austria.
4. Ehrlich, P. 1888. Charité-Annalen. **13**: 300.
5. Ehrlich, P. 1887. Arch. mikroskop. Anat. **13**: 263.
6. Michels, N. A. 1938. Handbook of Hematology. **1**: 339. Hoeber. New York.
7. Singer, K., B. Fisher & M. A. Perlstein. 1952. Blood. **7**: 577.
8. Isaacs, R. 1938. Handbook of Hematology. **1**: 15-42. Hoeber. New York.
9. Bernhard, W. 1952. Nature. **170**: 359.
10. Bloch, E. H. & A. Powell. 1952. Science. **115**: 46.
11. Sunderharagiati, B. & C. S. Wright. 1953. J. Clin. Invest. **32**: 979.
12. Ponder, E. 1948. Hemolysis and Related Phenomena: 115–160. Grune & Stratton. New York.
13. Bessis, M. 1949. Rev. Hematologie. **4**: 294.
14. Kruszyński, J. 1950. J. Physiol. London. **111**: 89.
15. Granick, S. 1949. Blood. **4**: 404.
16. Ralph, P. H. 1947. Anat. Record. **98**: 489.
17. Bloom, M. L. & G. B. Wislocki. 1950. Blood. **5**: 79.
18. Bunting, C. H. 1938. Handbook of Hematology. **1**: 162. Hoeber. New York.
19. Gordin, R. 1952. Acta Med. Scand. Suppl. **270**.
20. Kautz, J. & Q. B. DeMarsh. 1954. Blood. **9**: 24.
21. Bloom, W. 1938. Handbook of Hematology. **1**: 375. Hoeber. New York.
22. Petrakis, N. L. 1953. Blood. **8**: 905.
23. Hemplemann, L. H. & N. P. Knowlton, Jr. 1953. Blood. **8**: 524.
24. Ringoen, A. R. 1938. Handbook of Hematology. **1**: 181. Hoeber. New York.
25. Wislocki, G. B., H. Bunting & E. W. Dempsey. 1947. Anat. Record. **98**: 527.
26. Michels, N. A. 1938. Handbook of Hematology. **1**: 326. Hoeber. New York.
27. Ehrlich, P. 1891. Farbenanalytische Untersuchungen zur Histologie und Klinik des Blutes. Hirschwald. Berlin, Germany.
28. Rosenthal, N. 1938. Handbook of Hematology. **1**: 451. Hoeber. New York.
29. Dillard, G. H. L., G. Brecher & E. P. Cronkite. 1951. Proc. Soc. Exptl. Biol. Med. **78**: 796.
30. Schwarz, E. 1948. Arch. Path. **45**: 333.

31. Pisciotta, A. V., M. Stefanini & W. Dameshek. 1953. Blood. **8**: 703.
32. Braunsteiner, H., K. Fellinger & F. Pakesch. 1953. Blood. **8**: 916.
33. Sabin, F. R. 1921. Bull Johns Hopkins Hosp. **32**: 314.
34. Cunningham, R. S. & E. H. Tompkins. 1938. Handbook of Hematology. **1**: 555. Hoeber. New York.
35. Hall, B. E. 1938. Handbook of Hematology. **1**: 643. Hoeber. New York.
36. Schwind, J. L. 1950. Blood. **5**: 597.
37. Wintrobe, M. M. 1951. Clinical Hematology: 342 Lee & Febiger. Philadelphia, Pa.
38. Wislocki, G. B., J. J. Rheingold & E. W. Dempsey. 1949. Blood. **4**: 562.
39. Rheingold, J. J. & G. B. Wislocki. 1948. Blood. **3**: 641.
40. Wachstein, M. 1950. Proc. Soc. Exptl. Biol. Med. **73**: 306.
41. Bloom, M. L. & G. B. Wislocki. 1950. Blood. **5**: 79.
42. Kirkman, H. 1950. Am. J Anat. **86**: 91.
43. Ayres, W. W. & N. M. Starkey. 1950. Blood. **5**: 254.

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